The regulation of blood coagulation by protein S in the absence of activated protein C : from test tube to plasma

The Regulation of Blood Coagulation by Protein S in the Absence of Activated Protein C; from Test Tube to Plasma

Koenen, Robert R.

The Regulation of Blood Coagulation by Protein S in the Absence of Activated Protein C; from test tube to plasma. by Robert R. Koenen - Maastricht.
Thesis University Maastricht, 2005. With summary in Dutch.
ISBN 90-9018648-4

Omslag/ontwerp: Jennie Casu
Druk: PrintPartners Ipskamp BV, Enschede

The Regulation of Blood Coagulation by Protein S in the Absence of Activated Protein C; from Test Tube to Plasma

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. Mr. G.P.M.F. Mols,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen op
vrijdag 22 april 2005 om 12:00 uur.

door

Robert Ryan Koenen

PROMOTOR
Prof. Dr. J. Rosing

CO-PROMOTOR
Dr. T.M. Hackeng

BEOORDELINGSCOMMISSIE
Prof. Dr. M.J. Post, voorzitter
Prof. Dr. R.M. Bertina (Leids Universitair Medisch Centrum, Leiden)
Prof. Dr. H. ten Cate
Prof. Dr. M.P. van Dieijen-Visser
Prof. Dr. M.H. Prins

The work presented in this thesis was financed by program grant 902.26.210 from the Dutch Organization for Scientific Research (NWO).

Financial support by the Netherlands Heart Foundation and by the foundation Dr. Ir. van de Laar for the publication of this thesis is gratefully acknowledged.

"Curiouser and curiouser!"

Lewis Carroll (1832 - 1898)

Aan Mam en Pap.

Table of contents

Chapter 1

Basic concepts of blood coagulation.

Introduction

In suspensions with low cell densities, diffusion of oxygen and nutrients from the environment is sufficient for cell survival. Once a group of cells reaches a diameter of around 5 mm, diffusion alone is no longer sufficient and a transport system is needed for the efficient delivery of oxygen and nutrients, and the removal of waste products. In higher organisms this transport system has evolved into an intricate blood vessel network through which blood is pumped by the heart. This blood vessel network is a complex organ that is subject to continuous regulation. Blood pressure, blood flow and the volume of the vascular system for example are regulated by contraction of the blood vessels and the heart rate which are, themselves, also subject to complex regulatory mechanisms.

Blood fluidity is also tightly regulated. In case of injury, excessive blood loss is prevented by the hemostatic system which describes a complex interplay between blood cells (e.g. platelets), (sub-) endothelial cells and constituents of the blood plasma itself. Local arrest of blood flow falls under the definition of hemostasis. In plasma, a continuous equilibrium exists between mechanisms that drive and that inhibit coagulation. Uncontrolled coagulation is undesirable; since it may block the supply of blood to organs critical for the organism's survival and may potentially lead to death.

Primary hemostasis

The vascular endothelium is in constant contact with blood, and it plays an important role in the maintenance of blood flow by secreting particular substances. Prostacyclin[1] (PGI2) and nitric oxide[2] (NO) serve as vasorelaxators and inhibit platelet activation.

In addition, certain proteins (e.g. endothelium protein C receptor (EPCR), thrombomodulin (TM)) and heparan sulfates present on the endothelial surface serve to enhance the activity of anticoagulant regulators in plasma. Fibrinolysis is promoted through secretion of tissue plasminogen activator (tPA)[3].

In the case of endothelial damage or by the action of inflammatory stimuli, the endothelium secretes or exposes substances that promote vasoconstriction (endothelins), platelet aggregation (von Willebrand factor (vWF)) and blood coagulation (tissue factor (TF)). Upon endothelial damage, constituents of the extracellular matrix e.g. vWF, collagen and fibronectin are exposed and these substances cause platelet adhesion and activation. Interestingly, the substance responsible for platelet adhesion may differ at different flow conditions of the blood, i.e. at different shear rates. At high shear rates vWF, present in the subendothelium, promotes adhesion of platelets mediated by the receptor complex GPIb/V/IX and integrin $\alpha_{IIb}\beta_3$ on the platelet surface. Interaction of the platelet with collagen is mediated by platelet receptor GPVI and integrin $\alpha_2\beta_1$ and at high shear conditions initial binding of vWF to GPIb is required.

The initial platelet adhesion is followed by platelet activation and stable adhesion and these processes occur via complex intracellular signaling events. Platelets bound to the collagen/vWF matrix form a substrate for other platelets to adhere and thus form a thrombus. At low shear rates platelet/platelet interactions are mediated by binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$ and at higher shear rates platelet/platelet interactions occur via binding of vWF to GPIb. During thrombus growth continuous signaling between platelets occurs via messenger molecules like adenosine diphosphate (ADP), thromboxane A2 (TXA2) or thrombin and this signaling supports the process of stable thrombus formation. In addition, the phospholipid composition of the platelet surface changes upon activation.

The negatively charged phospholipid phosphatidylserine is exposed at the outer platelet surface and facilitates procoagulant enzymatic reactions of the humoral blood coagulation system which eventually leads to fibrin deposition at the site of injury.

The coagulation cascade

The coagulation cascade is characterized by sequential activation steps leading to the formation of thrombin, the enzyme that plays a central role in blood coagulation (Fig. 1). A key characteristic of the coagulation cascade is that the constituents, the coagulation factors, circulate as inactive precursor forms (zymogens) that can be activated through limited proteolysis. The activated proteases that participate in the blood coagulation cascade are all members of the family of serine proteases, which have a serine in their active sites.

The serine protease precursors prothrombin, factor VII, factor IX and factor X are synthesized in the liver and require vitamin K for proper biosynthesis. Vitamin K acts as a cofactor in the γ-glutamyl carboxylase-catalyzed conversion of glutamic acid to γ-carboxyglutamic acid (Gla), a process which has been shown to occur intracellularly, in the endoplasmatic reticulum[4]. The amino terminal part of the vitamin K-dependent clotting factors contains 10 to 12 Gla-residues and is thus referred to as Gla-domain. The Gla-domain is important for calcium-dependent binding of the vitamin K-dependent coagulation factors to membranes containing negatively charged phospholipids (such as those provided by activated platelets). Binding of calcium ions to the Gla-residues has been shown to induce a conformational change in the Gla-domain that causes exposure of hydrophobic amino acids that are important for phospholipid binding[5].

The efficiency of pro- and also anticoagulant reactions is increased by the two dimensional surface provided by negatively charged phospholipid membranes; the chance of collision is greater in two dimensions than in three dimensions and the active sites of coagulation proteases may be placed in a position favorable for reaction. In addition, non-enzymatic cofactors such as factors Va and VIIIa considerably increase the catalytic activity of factor Xa and IXa, respectively, and enhancement of binding to phospholipids appears to be one of the underlying mechanisms for this stimulation.

It was in the mid-sixties of the previous century, that blood coagulation was recognized as a cascade of sequential enzymatic reactions and the classical intrinsic and extrinsic pathways of blood coagulation were defined[6,7]. With the discovery of new intersections between both pathways the difference between the intrinsic and extrinsic pathways faded and nowadays, these classical pathways are rather referred to as the contact activation and tissue factor pathway, respectively (Fig. 1). The modern view of blood coagulation at present is more in terms of initiation and propagation phases (Fig. 1).

The tissue factor pathway is presently considered the physiological pathway of coagulation initiation. It is mediated by tissue factor (TF), a transmembrane protein of the cytokine receptor superfamily, which is present in certain cell types in the adventitia of blood vessels, but is also inducible in monocytes[8] and endothelial cells[9,10]. In the case of vascular damage, TF is exposed and binds factor VIIa, which circulates in trace amounts in plasma[11]. While free plasma factor VIIa is a poor activator of factor X, the catalytic efficiency of factor VIIa for factor X activation is greatly enhanced upon binding to TF[12]. In addition, the TF/factor VIIa complex is also able to activate factor IX, creating an amplification loop known as the "Josso loop"[13,14]. The low amounts of thrombin formed by factor Xa will activate factors VIII and V, and thrombin is also able to activate factor XI in the presence of negatively charged surfaces[15,16] (Fig. 1). Factor VIIIa will assemble with factor IXa, calcium and phospholipids to the so-called "intrinsic tenase complex", which activates factor X about five orders of magnitude more efficiently than factor IXa alone[17].

In a similar manner, the "prothrombinase complex" is formed by factor Xa, factor Va, calcium ions and phospholipids and this complex also possesses greatly enhanced catalytic efficiency of prothrombin activation compared with factor Xa alone[18]. The positive feedback loop provided by the activation of cofactors V and VIII by the first traces of thrombin that are formed results in a large burst of thrombin generation. Thrombin will convert fibrinogen into fibrin, which forms long polymers that are crosslinked to a stable fibrin network by factor XIIIa, a transglutaminase also activated by thrombin. The network of fibrin will further stabilize the initially formed platelet thrombus.

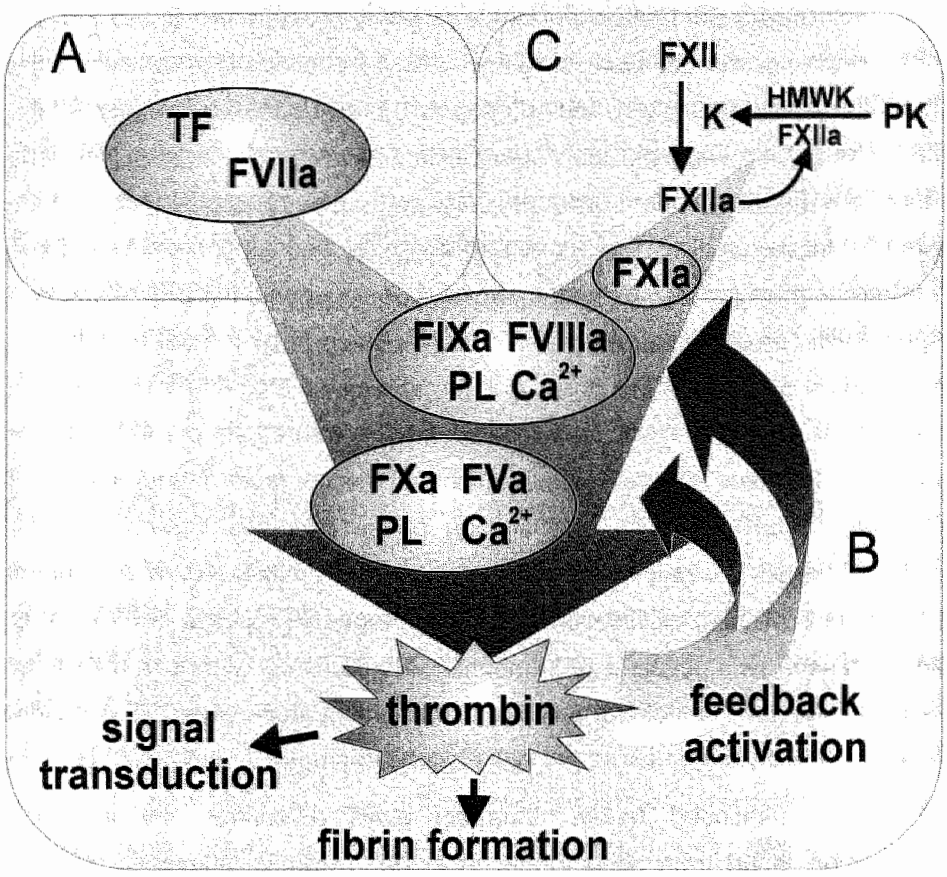

Figure 1. Schematic representation of the blood coagulation cascade.
Initiation of the blood coagulation cascade occurs via the tissue factor pathway or the contact activation pathway (initiation phase, panel A and panel C respectively). Once thrombin has been formed it exerts various functions such as platelet activation and conversion of fibrinogen into fibrin. Thrombin generation is propagated through feedback activation of factors V, VIII and XI, thus creating a feedback stimulatory loop (propagation phase, panel B).

Thrombin has multiple functions in hemostasis and the formation of fibrin may not be the most important one. Fibrin is formed at the end of the initiation phase when there is about 5-10 nM of thrombin present, while major thrombin generation occurs in the propagation phase after the formation of the fibrin clot[19]. This suggests that thrombin has more functions than just fibrin formation. For example, transgenic mice with null-mutations that exclude thrombin formation die before, or shortly after birth, because of fatal bleeding[20-22] whereas mice lacking fibrinogen survive but are unable to carry offspring[23]. Apparently, considerable amounts of thrombin need to be formed for effective hemostasis, rather than just fibrin. At high physiologic triggers, i.e. when large amounts of TF are present, sufficient thrombin is formed via the tissue factor pathway. However at low concentrations of tissue factor, amplification of thrombin formation via the propagation phase becomes important[24]. The aforementioned "Josso loop" may be crucial for this amplification (Fig. 1, panel B). The lack of this amplification during the propagation phase is believed to be the reason why people with hemophilia A or B (factor VIII or IX deficiency respectively) have bleeding problems, particularly in areas where TF is scarce (e.g. the joints).

As mentioned before, thrombin plays a central role in blood coagulation, apart from fibrin formation alone. Thrombin stimulates its own formation by creating a feedback loop, but thrombin may also downregulate its formation via activation of protein C. It can participate in certain signal transduction pathways such as those leading to platelet activation or inflammatory effects and thrombin may down-regulate the breakdown of fibrin (fibrinolysis) via activation of thrombin-activatable fibrinolysis inhibitor (TAFI).

The physiological importance of the contact activation pathway is not exactly clear, it may participate in some kind of host defense mechanism. The contact activation pathway is initiated through contact with foreign substances (e.g. glass or membranes of bacteria), negatively charged surfaces in particular. Contact activation is mediated by activated factor XII (factor XIIa) which activates prekallikrein (PK) to kallikrein (K) in the presence of high molecular weight kininogen (HMWK) (Fig. 1, panel C). Kallikrein can, in turn, activate factor XII and thus a positive feedback loop is formed. Factor XIIa activates factor XI and factor XIa subsequently activates factor IX eventually leading to thrombin formation.

Activation of prothrombin: the prothrombinase complex

Thrombin is a 36 kD protein, with two chains linked covalently via a disulfide bond. It contains several (exo)sites important for its function, such as the anion binding exosites 1 and 2 and the sodium binding site. Anion binding exosite 2, mediates binding to heparin[25] and anion binding exosite 1 interacts with e.g. fibrinogen[25], thrombomodulin[26] and the protease activated receptor 1 (PAR1)[27] and is also the target for particular venom components such as hirudin (from *Hirudo medicinalis*)[28,29] or bothrojaracin (from *Bothrops jararaca*)[30]. The anion binding exosites are also present in prothrombin, as less active pro-exosites.

Prothrombin is a 72 kD Gla-containing glycoprotein that is synthesized in the liver and circulates in plasma at a concentration of about 1.5 μM. Human prothrombin contains several peptide bonds that can be cleaved, but the sites at amino-acid positions Arg271 and Arg320 are essential for its activation by factor Xa (Fig. 2). In the absence of factor Va, cleavage of prothrombin by factor Xa mainly occurs at Arg271 releasing the Gla-containing fragment 1.2 and prethrombin 2, the latter being an inactive precursor of thrombin[18]. Successive cleavage of prethrombin 2 at Arg320 results in the formation of the active two chain α-thrombin. In the presence of factor Va, the order of cleavage changes.

Cleavage of prothrombin at Arg320 takes place first, resulting in the formation of an active 72 kD two chain thrombin variant called meizothrombin[18,31] (Fig. 2), which is rapidly converted into α-thrombin via cleavage at Arg271. As meizothrombin still contains the Gla-domain, it is able to bind phospholipids and has been reported to activate factor V[32] in the presence of phospholipid membranes. However, meizothrombin is almost inactive in the conversion of fibrinogen to fibrin[33], possibly because exosite 1 may not yet be in its mature form.

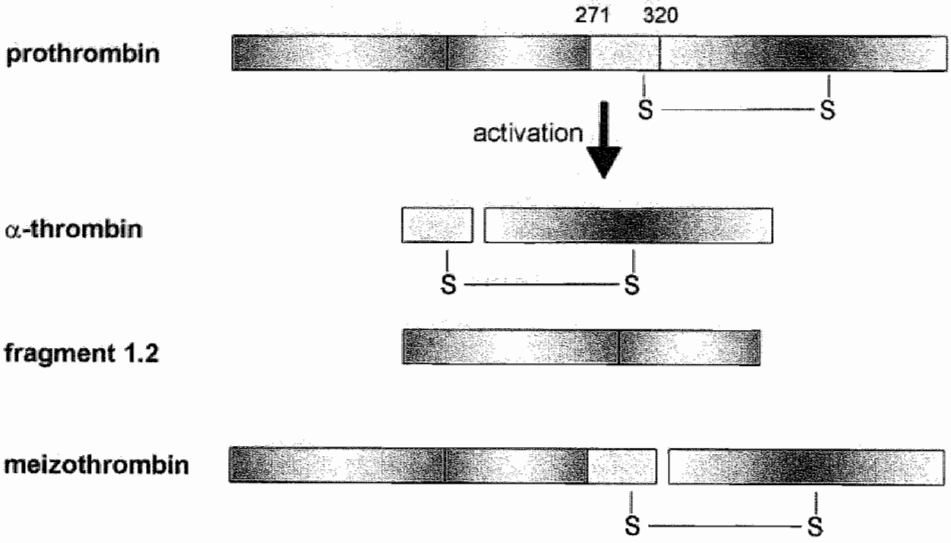

Figure 2. Schematic view of prothrombin activation.
Prothrombin is cleaved by factor Xa at two distinct sites. Cleavage at arginine 271 releases fragment 1.2, cleavage at arginine 320 results in catalytically active two chain enzyme (thrombin or meizothrombin). Autocleavage sites for thrombin are not shown in this scheme.

Factor V is a 330 kD glycoprotein that circulates in plasma at a concentration of approximately 25 nM. It contains several distinct domains (Fig. 3) and has homology with factor VIII. Thrombin is thought to interact with factor V via its anion binding exosites [34] and activates factor V via cleavage at three sites; Arg709, Arg1018 and Arg1545 (Fig. 3). In the presence of phospholipids, factor Xa is also able to activate factor V via cleavage at the same sites, albeit in a less efficient manner[35-37]. Complete cleavage of factor V at these sites by thrombin or factor Xa releases the B-domain and results in the active cofactor. Factor Va contains two chains, a heavy chain (105 kD) and a light chain (71-74 kD), the correct orientation of which is maintained by ionic calcium (Fig. 3). Under optimal conditions (in the presence of calcium ions and phospholipids), factor Va accelerates factor Xa-catalyzed prothrombin activation over 100,000-fold compared with factor Xa alone[18]. The exact mechanism through which factor Va accelerates prothrombin activation by factor Xa is not known. It is likely that the stimulating effect of factor Va is the sum of various enhancing effects. Factor Va enhances binding of factor Xa to negatively charged phospholipids[38], thereby increasing the Vmax of prothrombin activation. In addition, factor Va may induce conformational changes in the active site of factor Xa that increase the rate of macromolecular substrate conversion or alternatively, it may induce one or more substrate binding exosites in factor Xa. Recent data suggest that exosites present on factor Va and/or Xa in the prothrombinase complex may play an important role in the conversion of prothrombin into thrombin[39,40]. The proposed mode of action is that the exosites bind prothrombin and position the scissile bonds in prothrombin relative to the active site in a way favorable for cleavage. The substrate binding step will decrease Km while the internal positioning of the scissile bonds will mainly increase kcat of the prothrombin activation. However, the exosites involved in the process have not yet been identified.

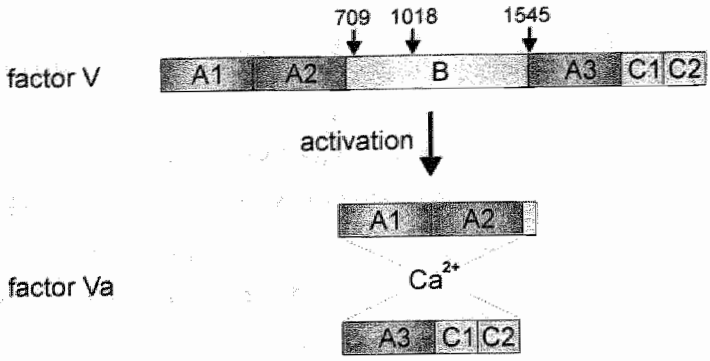

Figure 3. Schematic representation of factor V activation.
Thrombin or factor Xa activate factor V through cleavage at arginines 709, 1018 and 1545 resulting in the active two chain cofactor whose conformation is maintained by ionic calcium. The heavy chain consists of the A1 and A2 domains plus a small section of the B domain.

Regulation of blood coagulation.

A common property of enzymatic cascades is that considerable amplification of the initial stimulus is achieved with each zymogen activation step. Also in the case of the blood coagulation cascade, even a small initial procoagulant stimulus (e.g. TF or factor XIIa) may be amplified several orders of magnitude as a consequence of the successive zymogen activation steps. If this process was to be unregulated, plasma would quickly degenerate into a pandemonium of activated clotting factors. Fortunately, an intricate regulation system exists that prevents excessive activation of the procoagulant system and that localizes procoagulant reactions at the site of injury.

Several non-enzymatic protease inhibitors circulate in plasma and these inhibitors are in large molar excess relative to the activated enzymes. Among these inhibitors are for example antithrombin (AT), heparin cofactor II (HCII) and α_2-macroglobulin (α_2M). These inhibitors form stable yet reversible 1:1 complexes with activated coagulation factors.

Proteases in complex with AT or HCII are blocked at the active site while proteases inhibited by α_2M are trapped in a cage-like structure formed by α_2M; their active sites are still accessible by low molecular weight substrates [41]. AT is able to inhibit just about every relevant serine protease in the blood coagulation system (e.g. factors XIIa, XIa, IXa, Xa and thrombin) although its activity towards each protease is different. For example, freely circulating factor VIIa is poorly inhibited by AT whereas factor VIIa bound to TF, in turn, is more effectively inhibited [42,43]. In addition, the activity of AT is greatly enhanced by glycosaminoglycans such as heparan sulfates or heparin. Heparin binds to AT via a core pentasaccharide motif while thrombin binds to remaining part of the heparin chain and hence inhibitor and protease are brought together by heparin [44,45]. The stimulating action of heparin in the factor Xa inhibition by AT works in a different manner; binding of AT to the small pentasaccharide motif causes a conformational change in AT that increases factor Xa inhibition approximately 270-fold [45]. The action of HCII is primarily towards thrombin (but also cathepsin G and chymotrypsin) and heparin or dermatan sulfate is required for effective thrombin inhibition. However, its importance as anticoagulant is not well established. It may play a role as an extravascular thrombin inhibitor[46]. α_2-Macroglobulin is a broad range protease-inhibitor that may also play a role in the inhibition of the proteases involved in blood coagulation and fibrinolysis.

Another important inhibitor is tissue factor pathway inhibitor (TFPI) which has become a subject of ever increasing investigation during the past decade. TFPI is a (~40 kD) protease inhibitor with three Kunitz type domains, its free plasma concentration is about 2.5 nM[47]. The second Kunitz domain is responsible for the slow and relatively weak inhibition of factor Xa[48]. Binding of factor Xa to TFPI is required for effective inhibition of the TF/factor VIIa complex and once factor Xa is bound to TFPI, binding of TFPI/factor Xa to TF/factor VIIa occurs at nearly diffusion limited rate[49]. This binding step is mediated by the first Kunitz domain[48] and the resulting quaternary complex is nearly inactive in the activation of factor IX and X.

Apart from the non-enzymatic coagulation inhibitors, an enzymatic anticoagulant system also exists in the form of the protein C pathway. Protein C is a Gla-containing serine protease precursor that has a molecular weight of about 62 kD. Its plasma concentration is 65 nM and approximately 90% of protein C circulates in a two-chain form[50]. Protein C is effectively activated by thrombin bound to the endothelial thrombin-receptor thrombomodulin[51]. The resulting serine protease activated protein C (APC) plays an important role in the down-regulation of thrombin formation. The action of APC is complex due to the fact that APC inactivates both factor Va and VIIIa and each of these inactivation reactions has different cofactor requirements. Apart from having anticoagulant properties, APC also exerts anti-inflammatory effects (reviewed by Esmon[52]).

Protein S is a 75 kD vitamin K-dependent glycoprotein which bears considerable homology with the vitamin K-dependent serine proteases. However, it lacks a serine protease region. Instead, protein S has a C-terminal globular domain that shows extensive homology with rat androgen binding protein and sex hormone binding globulin (SHBG). This SHBG-like domain is thought to mediate various interactions with other proteins, one of which is C4b-binding protein (C4BP), a component of the classical complement cascade. C4BP contains six or seven alpha subunits (70 kD) and approximately 85% of circulating C4BP also contains one beta subunit (48 kD)[53]. The beta subunit of C4BP is responsible for the tight 1:1 interaction with protein S and the protein S/C4BP complex constitutes approximately 60% of protein S present in plasma. The remaining 40% of protein S is the molar surplus of protein S over beta chain containing C4BP[53] and circulates in a free form in plasma.

A well characterized function of protein S is its ability to act as a cofactor for APC in the inactivation of factor Va and VIIIa[54-56]. However, protein S also inhibits thrombin formation in an APC-independent fashion[57-59]. APC-cofactor function of protein S is largely lost upon binding of C4BP[60] while APC-independent activity is retained[59,61]. Since protein S, C4BP and the characterization of the APC-independent anticoagulant activity of protein S are topics of this thesis, a more extensive overview of these topics will be given in chapter 2.

APC inactivates factor Va (and VIIIa) via limited proteolysis. Factor Va is cleaved by APC at three main sites located in the heavy chain, Arg306, Arg506 and Arg679 (Fig. 4). Cleavage at Arg506 by APC occurs rapidly, resulting in a factor Va molecule with intermediate activity. Cleavage at Arg306 proceeds much slower and results in a virtually inactive factor Va molecule[62]. The APC-cleavage site at Arg679 has not been considered physiologically important. However, recently it has been shown that cleavage at Arg679 contributes to the inactivation of factor V variants Hong Kong and Cambridge, both of which cannot be cleaved by APC at position 306[63]. Efficient APC-catalyzed factor Va inactivation occurs in the presence of protein S. Protein S accelerates the APC-catalyzed cleavage at Arg306 approximately 20-fold while the rate of cleavage at Arg506 is increased by a modest twofold[64]. When particular phospholipids (such as glucosylceramide) are present, protein S also accelerates Arg506 cleavage by APC 5 to 13-fold[65,66]. Protein S has also been shown to abrogate protection of factor Va by factor Xa [67], which inhibits the cleavage at Arg506 in factor Va [64,67].

APC also inactivates factor VIIIa, but this inactivation proceeds rather inefficiently in the presence of only APC. In fact, factor VIIIa is quite unstable and the action of APC only modestly adds up to the spontaneous inactivation of factor VIIIa. The APC-catalyzed inactivation of factor VIIIa proceeds somewhat more rapidly in the presence of protein S or factor V and efficient APC-catalyzed factor VIIIa inactivation occurs when both protein S and factor V are present[68,69].

Cleavage of factor V by APC at Arg506 must occur before factor V can act as a cofactor for APC in factor VIIIa inactivation and this cofactor function is lost after cleavage by thrombin[68,70]. Analogous to APC-catalyzed factor Va inactivation, factor VIIIa is protected against APC-catalyzed inactivation by factor IXa and factor X. But in this case protein S is only able to abrogate the protective effect of factor X[71].

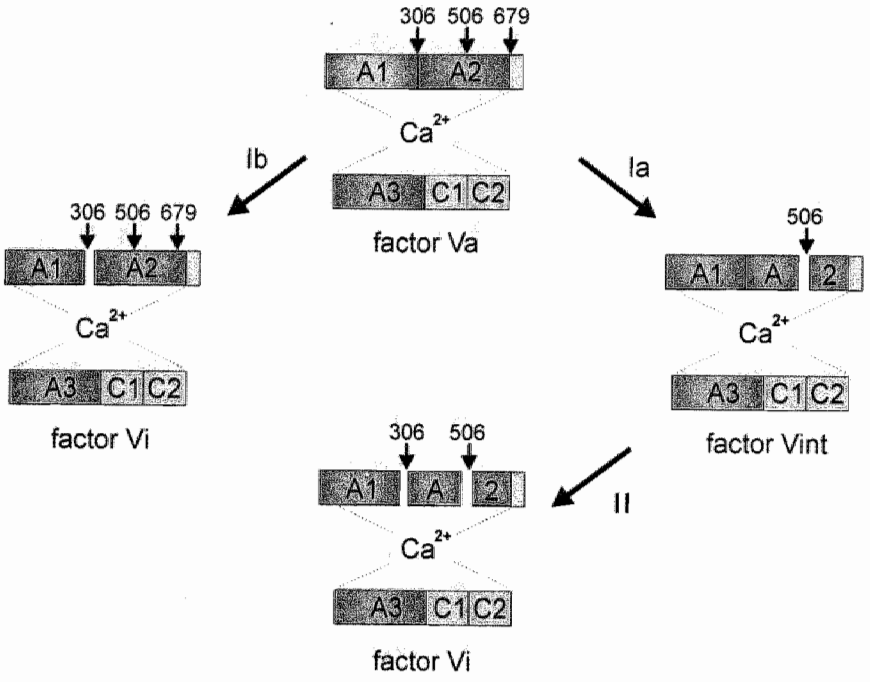

Figure 4. Schematic view of APC-catalyzed factor Va inactivation.
APC catalyzes cleavage at arginines 306, 506 and 679 in the heavy chain of factor Va. (Ia) Cleavage at Arg506 occurs with the highest rate resulting in a less active intermediate (factor Vint). (II) Cleavage at Arg306 results in a virtually inactive factor Va molecule (factor Vi). (Ib) Inactivation of factor Va may also occur directly via cleavage at Arg306. Protein S specifically accelerates cleavage at Arg306, but under particular conditions cleavage at Arg506 is also accelerated by protein S. Products resulting from cleavage at Arg679 have been omitted in this scheme.

The protein C pathway provides powerful feedback regulation of the coagulation cascade and the protein C pathway is in turn regulated by plasma inhibitors like protein C inhibitor or α_2M. In addition, the amount of free protein S may be regulated by β-chain-containing C4BP.

Besides mechanisms that regulate thrombin formation, the formation and breakdown of the fibrin network is also regulated. The formation of fibrin is controlled largely through the regulation of thrombin generation. The breakdown of fibrin, called fibrinolysis, is mainly initiated by the tissue-derived serine protease tissue plasminogen activator (tPA). Plasminogen is converted to plasmin by tPA and plasmin cleaves fibrin into small fragments. Plasminogen binds to fibrin via its lysine binding sites and fibrin serves as a kind of catalytic surface for the activation of plasminogen to plasmin. Fibrinolysis is regulated by protease inhibitors such as α_2-antiplasmin and plasminogen activator inhibitor 1 (PAI-1) and by a carboxypeptidase known as thrombin-activatable fibrinolysis inhibitor (TAFI). TAFI removes C-terminal ends of fibrin thus lowering the affinity for plasminogen. Hence, plasminogen activation by tPA does not efficiently occur on TAFI-cleaved fibrin. As its name states, TAFI is activated by thrombin. Thrombin in complex with thrombomodulin has been shown to be a better activator of TAFI than thrombin free in solution. High levels of thrombin favor TAFI activation and may thus result in a fibrin clot less prone to breakdown.

Genetic and acquired risk factors for thrombosis.

Thrombosis is considered to be a multifactorial disease in which a combination of genetic and/or environmental factors may, to a greater or lesser extent, contribute to the occurence of venous-thromboembolic events (VTE). Several risk factors, such as factor V_{Leiden} or the prothrombin G20210A mutation are well established whereas others like airplane travel are still a matter of debate. The severity of a risk factor may vary and some (if not all) risk factors may only predispose the carrier if present in combination with one or more other risk factors.

The marked presence of strong risk factors for thrombosis in an individual may be indicated by the term thrombophilia, meaning that the individual has an increased risk for venous thrombosis. Causes for thrombophilia may be either of genetic or acquired origin.

A well known cause for thrombosis is APC resistance which has been first described by Dahlbäck in 1993[72]. APC resistance is characterized by a poor response of a patient's plasma to the added anticoagulant action of APC. This finding suggested an impaired function of the protein C pathway. The observation that addition of purified factor V was able to correct APC resistance indicated an involvement of factor V, potentially as cofactor for APC. A major cause of APC resistance was later identified by several research groups as an amino acid substitution Arg 506 to Gln in factor V, the resulting factor V variant is presently known as factor V_{Leiden}. About 20-60% of patients with VTE were shown to have APC resistance and in 90% of the individuals with APC resistance factor V_{Leiden} has been found.

Factor V_{Leiden} is currently the best known genetic risk factor for VTE and is present in about 5% of the Caucasian population. Due to the aforementioned amino acid substitution, factor V_{Leiden} is less susceptible to inactivation by APC. In addition, APC is unable to initiate the cofactor function of factor V_{Leiden} in the APC-catalyzed factor VIIIa inactivation, as this requires cleavage at position 506. Although the factor V_{Leiden} mutation is a gain as well as a loss of function mutation, the effect is strongly procoagulant. The procoagulant role of factor V_{Leiden} as a factor Xa cofactor is enhanced because of decreased inactivation by APC. The anticoagulant role of factor V_{Leiden} as APC-cofactor, on the other hand, is disturbed because of the impaired activation of the cofactor function of factor V_{Leiden}. Although factor V_{Leiden} has been shown to be the major determinant of APC resistance, APC resistance also occurs in the absence of factor V_{Leiden} and has been shown to be an independent risk factor for VTE [73].

Besides the hereditary APC resistance caused by factor V_{Leiden}, acquired APC resistance may also develop during malignancy, systemic lupus erythematosus, pregnancy or oral contraceptive (OC) use. Acquired APC resistance as a consequence of OC use has been indicated as a major determinant of the increased risk of VTE that accompanies OC use. The use of third generation OC has been shown to cause greater APC resistance than the use of second generation OC and is associated with a higher risk of VTE[74,75].

A more recently discovered genetic risk factor for VTE, the prothrombin G20210A mutation, was identified in 1996[76]. This mutation is located in the 3' untranslated region of the prothrombin gene, possibly leading to an alternative poly-adenylation location and results in increased plasma levels of prothrombin. Carriers of the prothrombin mutation have a 2-7 fold higher risk of VTE compared with non-carriers, particularly in combination with other risk factors. High prothrombin levels themselves have also been found to be a risk factor for VTE and have been shown to cause APC resistance[77]. The exact reason why elevated prothrombin levels cause thrombophilia is not known at present.

Besides factor V_{Leiden} and the prothrombin G20210A mutation, thrombophilia may be caused by inherited deficiencies in anticoagulant proteins such as antithrombin, protein C and protein S. These deficiencies however account for no more than 5-10% of all VTE cases. Deficiencies in coagulation proteins may also occur during the normal lifespan of an individual. Well known examples are deficiencies as a result of liver failure or deficiency in vitamin K-dependent proteins as a consequence of vitamin K-deficiency or oral anticoagulation therapy.

Outline of this thesis

The interactions of protein S with other coagulation factors that are important for the expression of the APC-independent activities of protein S are not known. One objective of the project is to determine the residues on protein S critical for its APC-independent effect. While an impaired function of the protein C/protein S pathway is a well established risk-factor for venous thrombosis, the physiological importance of the APC-independent activity remains unclear. In order to establish the APC-independent effect of protein S as physiologically relevant, the effect of protein S in the absence of APC will be determined in its natural context, human plasma. Results from measurements performed in reconstituted systems using purified components and in plasma will be integrated into a general model describing the role of protein S in the regulation of thrombin formation.

The general introduction of this thesis is given in chapter 1. Chapter 2 is an introduction to protein S and C4BP. In chapters 3 and 4, a naturally existing variant of protein S, protein S Heerlen, is characterized either as recombinant (chapter 3) or as plasma-purified (chapter 4) protein. In chapter 5 an assay for the quantification of the APC-independent activity in plasma is described and in chapter 6, the activity of protein S is measured in a population of women using oral contraceptives and compared with men and women who were not using oral contraceptives.

References

1. Weksler BB, Marcus AJ, Jaffe EA. Synthesis of prostaglandin I2 (prostacyclin) by cultured human and bovine endothelial cells. Proc Natl Acad Sci U S A. 1977;74:3922-3926
2. Moncada S, Palmer RM, Higgs EA. The discovery of nitric oxide as the endogenous nitrovasodilator. Hypertension. 1988;12:365-372
3. Loskutoff DJ, Edgington TE. Synthesis of a fibrinolytic activator and inhibitor by endothelial cells. Proc Natl Acad Sci U S A. 1977;74:3903-3907
4. Bristol JA, Ratcliffe JV, Roth DA, Jacobs MA, Furie BC, Furie B. Biosynthesis of prothrombin: intracellular localization of the vitamin K-dependent carboxylase and the sites of gamma-carboxylation. Blood. 1996;88:2585-2593
5. Sunnerhagen M, Forsen S, Hoffren AM, Drakenberg T, Teleman O, Stenflo J. Structure of the Ca(2+)-free Gla domain sheds light on membrane binding of blood coagulation proteins. Nat Struct Biol. 1995;2:504-509
6. Davie EW, Ratnoff OD. Waterfall Sequence for Intrinsic Blood Clotting. Science. 1964;145:1310-1312
7. Macfarlane RG. An Enzyme Cascade in the Blood Clotting Mechanism, and Its Function as a Biochemical Amplifier. Nature. 1964;202:498-499
8. Gregory SA, Morrissey JH, Edgington TS. Regulation of tissue factor gene expression in the monocyte procoagulant response to endotoxin. Mol Cell Biol. 1989;9:2752-2755
9. Bevilacqua MP, Pober JS, Majeau GR, Cotran RS, Gimbrone MA, Jr. Interleukin 1 (IL-1) induces biosynthesis and cell surface expression of procoagulant activity in human vascular endothelial cells. J Exp Med. 1984;160:618-623
10. Colucci M, Balconi G, Lorenzet R, Pietra A, Locati D, Donati MB, Semeraro N. Cultured human endothelial cells generate tissue factor in response to endotoxin. J Clin Invest. 1983;71:1893-1896
11. Morrissey JH, Macik BG, Neuenschwander PF, Comp PC. Quantitation of activated factor VII levels in plasma using a tissue factor mutant selectively deficient in promoting factor VII activation. Blood. 1993;81:734-744
12. Silverberg SA, Nemerson Y, Zur M. Kinetics of the activation of bovine coagulation factor X by components of the extrinsic pathway. Kinetic behavior of two-chain factor VII in the presence and absence of tissue factor. J Biol Chem. 1977;252:8481-8488
13. Josso F, Prou-Wartelle O. Interaction of tissue factor and factor VII at the earliest phase of coagulation. Thromb Diath Haemorrh Suppl. 1965;17:35-44
14. Osterud B, Rapaport SI. Activation of factor IX by the reaction product of tissue factor and factor VII: additional pathway for initiating blood coagulation. Proc Natl Acad Sci U S A. 1977;74:5260-5264

15. Gailani D, Broze GJ, Jr. Factor XI activation in a revised model of blood coagulation. Science. 1991;253:909-912
16. Naito K, Fujikawa K. Activation of human blood coagulation factor XI independent of factor XII. Factor XI is activated by thrombin and factor XIa in the presence of negatively charged surfaces. J. Biol. Chem. 1991;266:7353-7358
17. van Dieijen G, Tans G, Rosing J, Hemker HC. The role of phospholipid and factor VIIIa in the activation of bovine factor X. J Biol Chem. 1981;256:3433-3442
18. Rosing J, Tans G, Govers-Riemslag JW, Zwaal RF, Hemker HC. The role of phospholipids and factor Va in the prothrombinase complex. J Biol Chem. 1980;255:274-283
19. Brummel KE, Paradis SG, Butenas S, Mann KG. Thrombin functions during tissue factor-induced blood coagulation. Blood. 2002;100:148-152
20. Sun WY, Witte DP, Degen JL, Colbert MC, Burkart MC, Holmback K, Xiao Q, Bugge TH, Degen SJ. Prothrombin deficiency results in embryonic and neonatal lethality in mice. Proc Natl Acad Sci U S A. 1998;95:7597-7602
21. Dewerchin M, Liang Z, Moons L, Carmeliet P, Castellino FJ, Collen D, Rosen ED. Blood coagulation factor X deficiency causes partial embryonic lethality and fatal neonatal bleeding in mice. Thromb Haemost. 2000;83:185-190
22. Cui J, O'Shea KS, Purkayastha A, Saunders TL, Ginsburg D. Fatal haemorrhage and incomplete block to embryogenesis in mice lacking coagulation factor V. Nature. 1996;384:66-68
23. Suh TT, Holmback K, Jensen NJ, Daugherty CC, Small K, Simon DI, Potter S, Degen JL. Resolution of spontaneous bleeding events but failure of pregnancy in fibrinogen-deficient mice. Genes Dev. 1995;9:2020-2033
24. Cawthern KM, van 't Veer C, Lock JB, DiLorenzo ME, Branda RF, Mann KG. Blood coagulation in hemophilia A and hemophilia C. Blood. 1998;91:4581-4592
25. Tulinsky A. Molecular Interactions of Thrombin. Semin Thromb Hemost. 1996;22:117-124
26. Wu QY, Sheehan JP, Tsiang M, Lentz SR, Birktoft JJ, Sadler JE. Single amino acid substitutions dissociate fibrinogen-clotting and thrombomodulin-binding activities of human thrombin. Proc Natl Acad Sci U S A. 1991;88:6775-6779
27. Vu TK, Wheaton VI, Hung DT, Charo I, Coughlin SR. Domains specifying thrombin-receptor interaction. Nature. 1991;353:674-677
28. Braun PJ, Dennis S, Hofsteenge J, Stone SR. Use of site-directed mutagenesis to investigate the basis for the specificity of hirudin. Biochemistry. 1988;27:6517-6522
29. Stone SR, Braun PJ, Hofsteenge J. Identification of regions of alpha-thrombin involved in its interaction with hirudin. Biochemistry. 1987;26:4617-4624

30. Arocas V, Zingali RB, Guillin MC, Bon C, Jandrot-Perrus M. Bothrojaracin: a potent two-site-directed thrombin inhibitor. Biochemistry. 1996;35:9083-9089
31. Krishnaswamy S, Church WR, Nesheim ME, Mann KG. Activation of human prothrombin by human prothrombinase. Influence of factor Va on the reaction mechanism. J Biol Chem. 1987;262:3291-3299
32. Tans G, Nicolaes GA, Thomassen MC, Hemker HC, van Zonneveld AJ, Pannekoek H, Rosing J. Activation of human factor V by meizothrombin. J Biol Chem. 1994;269:15969-15972
33. Doyle MF, Mann KG. Multiple active forms of thrombin. IV. Relative activities of meizothrombins. J Biol Chem. 1990;265:10693-10701
34. Esmon CT, Lollar P. Involvement of thrombin anion-binding exosites 1 and 2 in the activation of factor V and factor VIII. J Biol Chem. 1996;271:13882-13887
35. Foster WB, Nesheim ME, Mann KG. The factor Xa-catalyzed activation of factor V. J Biol Chem. 1983;258:13970-13977
36. Monkovic DD, Tracy PB. Activation of human factor V by factor Xa and thrombin. Biochemistry. 1990;29:1118-1128
37. Thorelli E, Kaufman RJ, Dahlback B. Cleavage requirements for activation of factor V by factor Xa. Eur J Biochem. 1997;247:12-20
38. Krishnaswamy S. Prothrombinase complex assembly. Contributions of protein-protein and protein-membrane interactions toward complex formation. J Biol Chem. 1990;265:3708-3718
39. Boskovic DS, Krishnaswamy S. Exosite binding tethers the macromolecular substrate to the prothrombinase complex and directs cleavage at two spatially distinct sites. J Biol Chem. 2000;275:38561-38570
40. Krishnaswamy S, Betz A. Exosites determine macromolecular substrate recognition by prothrombinase. Biochemistry. 1997;36:12080-12086
41. Ganrot PO. Inhibition of plasmin activity by alpha-2-macroglobulin. Clin Chim Acta. 1967;16:328-329
42. Shigematsu Y, Miyata T, Higashi S, Miki T, Sadler JE, Iwanaga S. Expression of human soluble tissue factor in yeast and enzymatic properties of its complex with factor VIIa. J Biol Chem. 1992;267:21329-21337
43. Lawson JH, Butenas S, Ribarik N, Mann KG. Complex-dependent inhibition of factor VIIa by antithrombin III and heparin. J Biol Chem. 1993;268:767-770
44. Choay J, Petitou M, Lormeau JC, Sinay P, Casu B, Gatti G. Structure-activity relationship in heparin: a synthetic pentasaccharide with high affinity for antithrombin III and eliciting high anti-factor Xa activity. Biochem Biophys Res Commun. 1983;116:492-499
45. Olson ST, Bjork I, Sheffer R, Craig PA, Shore JD, Choay J. Role of the antithrombin-binding pentasaccharide in heparin acceleration of antithrombin-proteinase reactions. Resolution of the antithrombin conformational change contribution to heparin rate enhancement. J Biol Chem. 1992;267:12528-12538

46. McGuire EA, Tollefsen DM. Activation of heparin cofactor II by fibroblasts and vascular smooth muscle cells. J Biol Chem. 1987;262:169-175
47. Novotny WF, Brown SG, Miletich JP, Rader DJ, Broze GJ, Jr. Plasma antigen levels of the lipoprotein-associated coagulation inhibitor in patient samples. Blood. 1991;78:387-393
48. Girard TJ, Warren LA, Novotny WF, Likert KM, Brown SG, Miletich JP, Broze GJ, Jr. Functional significance of the Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor. Nature. 1989;338:518-520
49. Baugh RJ, Broze GJ, Jr., Krishnaswamy S. Regulation of Extrinsic Pathway Factor Xa Formation by Tissue Factor Pathway Inhibitor. J. Biol. Chem. 1998;273:4378-4386
50. Miletich J, Leykam F, Broze G, Jr. Detection of a single chain protein C in human plasma. Blood. 1983;62:(Abstract)
51. Esmon CT, Owen WG. Identification of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. Proc Natl Acad Sci U S A. 1981;78:2249-2252
52. Esmon CT. The Anticoagulant and Anti-inflammatory Roles of the Protein C Anticoagulant Pathway. Journal of Autoimmunity. 2000;15:113-116
53. Griffin J, Gruber A, Fernandez J. Reevaluation of total, free, and bound protein S and C4b-binding protein levels in plasma anticoagulated with citrate or hirudin. Blood. 1992;79:3203-3211
54. Walker FJ. Regulation of activated protein C by a new protein. A possible function for bovine protein S. J Biol Chem. 1980;255:5521-5524
55. Walker FJ, Chavin SI, Fay PJ. Inactivation of factor VIII by activated protein C and protein S. Arch Biochem Biophys. 1987;252:322-328
56. Koedam JA, Meijers JC, Sixma JJ, Bouma BN. Inactivation of human factor VIII by activated protein C. Cofactor activity of protein S and protective effect of von Willebrand factor. J Clin Invest. 1988;82:1236-1243
57. Heeb MJ, Mesters RM, Tans G, Rosing J, Griffin JH. Binding of protein S to factor Va associated with inhibition of prothrombinase that is independent of activated protein C. J Biol Chem. 1993;268:2872-2877
58. Heeb MJ, Rosing J, Bakker HM, Fernandez JA, Tans G, Griffin JH. Protein S binds to and inhibits factor Xa. Proc Natl Acad Sci U S A. 1994;91:2728-2732
59. Hackeng TM, van 't Veer C, Meijers JC, Bouma BN. Human protein S inhibits prothrombinase complex activity on endothelial cells and platelets via direct interactions with factors Va and Xa. J Biol Chem. 1994;269:21051-21058
60. Dahlbäck B. Inhibition of protein Ca cofactor function of human and bovine protein S by C4b-binding protein. J Biol Chem. 1986;261:12022-12027

61. Koenen RR, Tans G, van Oerle R, Hamulyak K, Rosing J, Hackeng TM. The APC-independent anticoagulant activity of protein S in plasma is decreased by elevated prothrombin levels due to the prothrombin G20210A mutation. Blood. 2003;102:1686-1692
62. Nicolaes GA, Tans G, Thomassen MC, Hemker HC, Pabinger I, Varadi K, Schwarz HP, Rosing J. Peptide bond cleavages and loss of functional activity during inactivation of factor Va and factor VaR506Q by activated protein C. J Biol Chem. 1995;270:21158-21166
63. Norstrom E, Thorelli E, Dahlback B. Functional characterization of recombinant FV Hong Kong and FV Cambridge. Blood. 2002;100:524-530
64. Rosing J, Hoekema L, Nicolaes GA, Thomassen MC, Hemker HC, Varadi K, Schwarz HP, Tans G. Effects of protein S and factor Xa on peptide bond cleavages during inactivation of factor Va and factor VaR506Q by activated protein C. J Biol Chem. 1995;270:27852-27858
65. Norstrom EA, Steen M, Tran S, Dahlback B. Importance of protein S and phospholipid for activated protein C-mediated cleavages in factor Va. J Biol Chem. 2003;278:24904-24911
66. Deguchi H, Fernandez JA, Griffin JH. Neutral glycosphingolipid-dependent inactivation of coagulation factor Va by activated protein C and protein S. J Biol Chem. 2002;277:8861-8865
67. Solymoss S, Tucker MM, Tracy PB. Kinetics of inactivation of membrane-bound factor Va by activated protein C. Protein S modulates factor Xa protection. J Biol Chem. 1988;263:14884-14890
68. Shen L, Dahlbäck B. Factor V and protein S as synergistic cofactors to activated protein C in degradation of factor VIIIa. J Biol Chem. 1994;269:18735-18738
69. Varadi K, Rosing J, Tans G, Pabinger I, Keil B, Schwarz HP. Factor V enhances the cofactor function of protein S in the APC-mediated inactivation of factor VIII: influence of the factor VR506Q mutation. Thromb Haemost. 1996;76:208-214
70. Thorelli E. Mechanisms that regulate the anticoagulant function of coagulation factor V. Scand J Clin Lab Invest Suppl. 1999;229:19-26
71. O'Brien LM, Mastri M, Fay PJ. Regulation of factor VIIIa by human activated protein C and protein S: inactivation of cofactor in the intrinsic factor Xase. Blood. 2000;95:1714-1720
72. Dahlback B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. Proc Natl Acad Sci U S A. 1993;90:1004-1008
73. de Visser MC, Rosendaal FR, Bertina RM. A reduced sensitivity for activated protein C in the absence of factor V Leiden increases the risk of venous thrombosis. Blood. 1999;93:1271-1276
74. Rosing J, Tans G, Nicolaes GA, Thomassen MC, van Oerle R, van der Ploeg PM, Heijnen P, Hamulyak K, Hemker HC. Oral contraceptives and venous thrombosis: different sensitivities to activated protein C in women using second- and third-generation oral contraceptives. Br J Haematol. 1997;97:233-238

75. Vandenbroucke JP, Helmerhorst FM, Bloemenkamp KW, Rosendaal FR. Third-generation oral contraceptive and deep venous thrombosis: from epidemiologic controversy to new insight in coagulation. Am J Obstet Gynecol. 1997;177:887-891
76. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood. 1996;88:3698-3703
77. Legnani C, Cosmi B, Valdre L, Boggian O, Bernardi F, Coccheri S, Palareti G. Venous thromboembolism, oral contraceptives and high prothrombin levels. J Thromb Haemost. 2003;1:112-117

Chapter 2

Structure and function of protein S

Introduction

Protein S is a vitamin K-dependent regulator of blood coagulation. It was first purified from human plasma by Di Scipio and coworkers in 1977 and arbitrarily named protein S, presumably after the city of its discovery (Seattle)[1]. At the time of its discovery, no function was ascribed to protein S. Later, in 1980 it was found by Walker that protein S acts as a non-enzymatic cofactor for APC in the inactivation of factor Va[2]. A year later, the high molecular weight complex between protein S and C4BP was described by Dahlbäck and Stenflo[3]. The importance of the anticoagulant function of protein S was emphasized by the description of VTE in families and patients with protein S deficiency in 1984[4-6]. In the late 1980s, protein S was also found to be a cofactor of APC in the factor VIIIa degradation[7,8]. New activities of protein S were described during the previous decade: the APC-independent anticoagulant effects of protein S were discovered[9-11] and protein S was found to be a ligand for certain receptor-like tyrosine kinases[12]. Recently, it was shown that protein S possesses anti-apoptotic and anti-inflammatory properties in a mouse ischemia/reperfusion model of stroke[13]. In addition, it was demonstrated that protein S enhances the uptake of apoptotic cells by macrophages[14]. Thus, protein S is emerging from a humble cofactor to a protein with multiple functions in coagulation, inflammation and apoptosis.

Biology of protein S and C4BP

Protein S is a 75 kDa glycoprotein and is a member of the γ-carboxyglutamic acid (Gla)-containing coagulation proteins, which require vitamin K for proper biosynthesis. Two genes, PROS1 and PROSP, which are located on chromosome 3, contain sequence data for protein S[15-17]. PROSP is a pseudo-gene, which is highly homologous to PROS1 but is not transcribed. The PROS1 gene contains 15 exons and codes for the protein S precursor. Protein S is synthesized by a variety of tissues e.g. liver [18], lung [19,20], endothelial cells [21-23] and protein S has also been found in osteoblasts [24] and the testes [25].

Activated platelets [26,27] and Interleukin-4 (IL-4) activated primary T-cells [28] have also been found to express protein S. Protein S is synthesized as a precursor with a signal peptide and a propeptide necessary for secretion and γ-carboxylation[16,29,30].

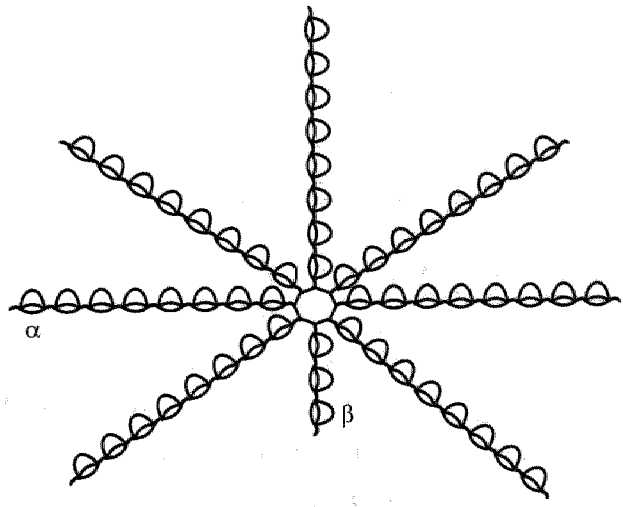

Figure 1: Schematic representation of C4b binding protein (C4BP).
The α-chain of C4BP is built of six to eight complement control protein (CCP) domains and the β-chain consist of three CCPs. The α and β-chains are covalently connected to a central core via disulfide bridges. The binding site for protein S is located on CCP1 and CCP2 of the β-chain. The α-chains of C4BP contain binding domains for several molecules and pathogenic microorganisms.

Human C4BP is a large molecule (570 kDa) which is composed of 6-8 α-subunits and is a regulator of the classical complement system. It has been found to be a cofactor of factor I in the degradation of C4b[31]. A novel subunit of C4BP, the β-subunit, was discovered that has been shown to mediate binding of protein S[32]. The α-chain and β-chain of human C4BP are expressed from two separate genes C4BPA and C4BPB respectively, both located in close proximity on chromosome 1 and the α- and β-chains are expressed in the liver [33].

The β-chain of C4BP (but not the α-chain) is also expressed in ovaries[34]. This β-chain of C4BP contains three so-called complement control protein (CCP) domains[35], similar to the longer α-chains that contain eight CCPs[36] (Fig. 1). The α-chains bind several ligands such as serum amyloid P (SAP), C4b, C3b and low density lipoprotein receptor-related protein (LRP) but also have affinity for several pathogenic microorganisms[37,38]. Recently, C4BP has been found to activate B-cells through the CD40 receptor[39]. Binding sites for protein S have been identified on CCP1 and CCP2 of the β-chain while CCP3 did not appear to contribute to the interaction with protein S [40,41].

Structure and interactions of protein S

Mature, processed protein S consists of multiple domains to which specific functions are ascribed (Fig. 2). The Gla-domain of protein S contains 11 Gla-residues and is responsible for calcium ion-dependent phospholipid binding [42]. Calcium ions have been shown to induce a conformational change in the Gla-domain of prothrombin, increasing its binding to phospholipids [43,44] and this will likely occur in protein S as well. Incomplete carboxylation of the Gla-domain (e.g. due to oral anticoagulant treatment) or mutations in the Gla-domain lead to dysfunctional protein S [45,46].

The Gla-domain is followed by a short aromatic stack and a thrombin sensitive region (TSR) (Fig. 2). The TSR contains three arginine residues at positions 49, 60 and 70 which are sensitive to cleavage by thrombin [47,48], factor Xa [49] and Protac C [50]. Thrombin has been shown to cleave protein S at Arg49 and Arg70 only in the absence of calcium-ions (Ca^{2+}) [51]. Unlike thrombin, factor Xa is able to cleave protein S at Arg60 in the presence of Ca^{2+} and phospholipids [49]. Protac C has recently been shown to cleave protein S at Arg70 [50]. Protein S cleaved in the TSR is inactive as a cofactor for activated protein C (APC) [52]. About 10-20% of total protein S in plasma appears to circulate in the cleaved form [52,53]. Increased levels of cleaved protein S have been observed in patients with disseminated intravascular coagulation (DIC) [53,54] and decreased levels (10%) in patients receiving oral anticoagulants [53]. The significance of these findings is not yet clear.

The TSR is followed by four Epidermal Growth Factor (EGF)-like modules (Fig. 2). The first EGF-like module (EGF 1) contains a β-hydroxyaspartic acid residue while the other three EFG domains contain β-hydroxyasparagine [55]. EGF 2-4 have been reported to bind Ca^{2+} [56], the highest Ca^{2+}-binding potential being located in EGF 3 and 4 [57,58]. A high affinity Ca^{2+}-binding site has been identified in EGF 4 (K_d=1 μM) and a lower affinity binding site in EGF 3 (K_d=4.8 mM) [59]. The EGF-like modules have been shown to be important for protein S function and have been identified to interact with APC and factor Xa. EGF-like module pair 3-4, for example, was shown to interact with factor Xa whereas no interaction was observed with EGF 3 or 4 alone [59]. A patient with recurrent venous thrombosis carried an inactive mutant protein S lacking the EGF 1 module [60]. Synthetic EGF 1 as well as EGF-like module pairs 1-3 and 1-4 have been shown to inhibit the APC-cofactor activity of protein S [61,62]. Thus, EGF 1 appears to be responsible for the interaction with APC and essential for the expression of APC-cofactor activity. However, protein S Tokushiwa, which contains a point mutation (Lys 155 → Glu) in the second EGF-like module did neither bind to factor Xa nor to APC and consequently, no APC-cofactor activity and prothrombinase inhibition were observed [63,64]. The importance of EGF 2 is further indicated by a recent study showing that recombinant protein S lacking EGF 2 is also inactive as a cofactor for APC [65].

Unlike the other Gla-containing coagulation factors, protein S does not possess a serine protease region. Instead, protein S contains a large globular region which has considerable sequence homology with the sex hormone binding globulin (SHBG) (Fig. 2), a plasma protein that circulates as a homodimer and is responsible for the transport of sex hormones [66,67]. The SHBG-like region of protein S consists of two laminin G (LG) modules and the second LG module contains three N-linked glycosylations. The LG domains, which were named after repeated globular structures in laminin, an extracellular matrix protein. The LG structure can be assigned to the pentraxin family [68].

Structure predictions[67] and crystal structure analysis of LG regions 4 and 5 of laminin[69] have indicated that these domains were most homologous to the LG domains of protein S, SHBG and also Gas6 (a protein that shares homology with protein S). In addition, the crystal structure of the N-terminal part of human recombinant SHBG, which is most homologous to protein S, has recently been resolved[70]. Based on the structural data obtained from human SHBG and laminin LG4 and 5 domains, Villoutreix et al. constructed a three-dimensional model of the protein S SHBG-like region using homology modelling[71]. The model yielded useful structural insights about the effects of naturally occurring point mutations and about C4BP and calcium binding.

The SHBG domain of protein S is thought to mediate a variety of interactions. The high affinity interaction of protein S to C4BP occurs via binding of the SHBG-like domain to C4BP[72,73]. Several sites important for the interaction have been identified in the SHBG-like domain of the protein S molecule. Using a synthetic peptide that was able to inhibit the binding of protein S to C4BP, Walker has shown that residues in the LG2 module 605-614 located in the loop located nearest to the C-terminus were involved in the interaction[74]. A mutagenesis study of protein S confirmed the importance of the region around 605-614 for C4BP binding[75], but studies using peptides comprising residues overlapping region 605-614 showed no effect on the protein S / C4BP interaction[76,77]. Thus, other regions may be involved in the interaction. Peptides containing residues 413-434 of the LG1 module and residues 447-460 in the LG2 module of protein S[76,77], have been shown to interfere with the protein S / C4BP interaction. Mutagenesis studies of protein S in combination with binding studies using synthetic peptide homologues and monoclonal antibodies further narrowed down these regions to residues 423-429[78] and 453-460[79] respectively. The distribution of these potential binding sites over the two LG modules of protein S corresponded well with the observation that both LG domains of protein S were necessary for stable binding of protein S to C4BP[80].

Homology modeling of the SHBG-like domain of protein S revealed that the important regions were located near the junction of the two LG modules and that the groove present at this interface may form a potential binding pocket for the CCP1 domain of the β-subunit of C4BP [71,79]. An interesting property of the interaction of protein S with C4BP is that it is Ca^{2+} dependent [81]. The dissociation constant of the complex decreases ~65 fold from 6.5 nM in the absence of Ca^{2+}, to 100 pM if Ca^{2+} is present [72]. Interestingly, the presence of calcium ions only appeared to have a major effect on the dissociation rate of the interaction of protein S with C4BP [72]. Although homology modeling has revealed a putative calcium binding site in the SHBG-like domain of protein S [71], the influence of this site has not yet been tested so it is still unclear whether this site is important for the interaction. Unfortunately, the C-terminal residues 632-635 in the second LG domain of protein S were not included in the model because these residues were not present in the modeling templates [71]. These C-terminal residues are interesting because region 621-635 has been identified to be essential for interaction of protein S with FVa [82].

The SHBG-like domain of protein S has also been shown to mediate the interaction of protein S with the receptor tyrosine kinase Tyro3/Sky [83,84]. The importance of this interaction is not yet known because the interaction of human protein S with human Sky does not lead to activation of the receptor [85].

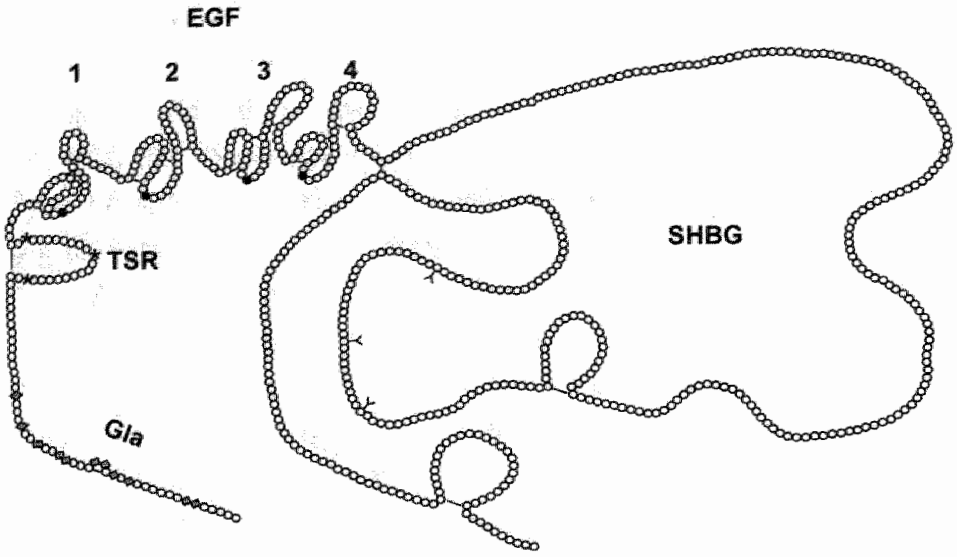

Figure 2: Modular structure of protein S.
Protein S consists of distinct modules: the N-terminal gamma-carboxyglutamic acid domain (Gla) with the Gla residues are shown as grey diamonds, the thrombin sensitive region (TSR) with residues susceptible to thrombin or factor Xa cleavage are displayed as asterisks, the four epidermal growth factor (EGF)-like domains with beta-hydroxylated Asp or Asn residues are marked in black and the sex hormone binding globulin (SHBG)-like region with N-linked glycosylated Asn residues are marked with "Y". The partition of the SHBG region in LG domains has been omitted in this figure.

Mechanism of APC cofactor function of protein S

Although enzyme/cofactor complexes are common in blood coagulation, the mechanisms by which cofactors accelerate catalysis are still largely unclear. The catalysis of factor Va inactivation by the protein S/APC complex has been the subject for extensive investigation. This catalysis is characterized by multiple cleavages and protein S preferentially enhances the factor Va-cleavage rate of R306 by APC. Therefore, the overall mechanism of the APC-cofactor activity of protein S is likely to be the result of several partial enhancing mechanisms.

One of these partial mechanisms has already been identified as early as 1981. Protein S was shown to enhance binding of APC to phospholipids and no enhancement of factor Va inactivation was observed in the absence of phospholipids[86].

Another more recently described effect of protein S is the relocation of the active site of APC (Fig. 3). Fluorescence resonance energy transfer (FRET) studies using active site labeled APC showed that the distance of the closest approach of the active site to the phospholipid membrane, decreased from 94 Å in the absence of protein S to 84 Å in the presence of protein S[87]. Interestingly, relocation of the APC active site could also occur in the presence of synthetic EGF1[88] of protein S. In addition, a recombinant chimeric APC with the Gla-domain of prothrombin, also showed a decreased distance of the active site to the phospholipid membrane (89 Å) and this APC variant was highly active in the absence of protein S. The rate of factor Va inactivation by the APC/prothrombin chimera was unaffected by the presence of protein S[89,90].

To date, not much is known regarding the mechanism of the acceleration by protein S of the APC-catalyzed factor VIIIa inactivation, but similar mechanisms may underlie the effect of protein S.

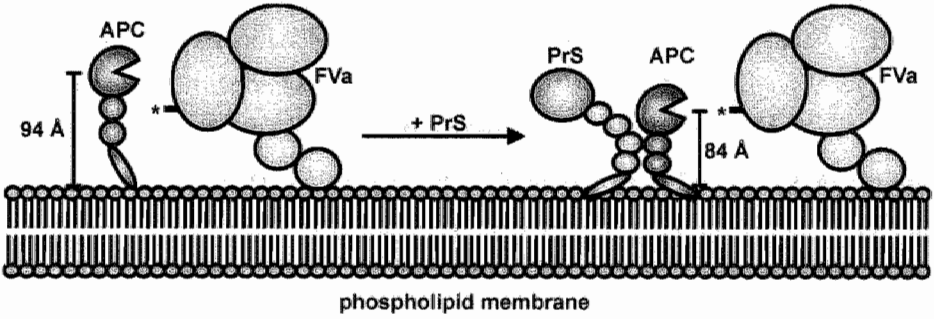

Figure 3: Active site displacement of APC by protein S.

The active site of APC is 94 Å away from the plane of the phospholipid membrane (left). In the presence of protein S (PrS), the position of the active site changes to 84 Å relative to the phospholipid surface (right). It is hypothesized that the active site of APC is placed at the same level as the target cleavage site at Arg306 (marked by *) in factor Va (FVa), via this active site displacement[90]. The translational motion of the active site of APC was calculated assuming a κ^2 of 2/3.

APC-independent anticoagulant activity of protein S

In 1988, a curious anticoagulant effect of protein S was described by Mitchell et al. Protein S, affinity purified using a monoclonal antibody, appeared to be modified in such way that it obtained anticoagulant activity in the absence of APC [91]. This modified form was found to dose dependently prolong the clotting time of alumina absorbed plasma triggered with factor Xa and was found to inhibit prothrombinase in a purified system. Protein S appeared to be a competitive inhibitor of factor Xa-catalyzed prothrombin activation. Interestingly, pre-incubation with the monoclonal antibody transformed normal protein S into the modified form and the authors speculated about the possibility of "activation" of plasma protein S to this modified form with APC-independent activity *in vivo*.

Later it was shown that plasma-derived protein S, which has not been in contact with antibodies, also inhibits prothrombinase activity in the absence of APC [9-11]. Protein S has been shown to bind to factor Va and factor Xa, thereby inhibiting prothrombin activation. It was also demonstrated by Heeb et al. that protein S competes with both prothrombin and factor Xa for binding sites on factor Va [10] and that protein S acts as a non-competitive inhibitor of the factor Xa activity [9]. Consequently, a pattern of mixed inhibition of prothrombinase by protein S was observed and the extent of this inhibition was independent over a large range of phospholipid concentrations [10] and has also been demonstrated on platelets and cultured endothelial cells [9]. Binding to phospholipids is important for the ability of protein S to inhibit prothrombinase, since a monoclonal antibody directed against the Gla-domain of protein S almost completely abolished prothrombinase inhibition [9]. Interestingly, the protein S / C4BP complex and protein S cleaved in the TSR also inhibited prothrombinase, albeit in a less potent manner than free protein S, probably because of the inability of these forms to bind to factor Va and factor Xa, respectively [9].

Further evidence for an APC-independent activity of protein S was obtained via a study observing prothrombin activation of heparinized plasma perfused over endothelial cells [92]. Perfusion of protein S-depleted plasma over tumor necrosis factor (TNFα) activated endothelial cells resulted in a 5- to 25-fold increase of prothrombin activation compared to normal plasma. Perfusion of protein C-depleted plasma, on the other hand, did not result in elevated prothrombin activation. The authors therefore concluded that the anticoagulant effect of protein S was APC-independent [92]. Unfortunately, thrombin formation in the perfusion model used in the aforementioned study appeared to be independent of the intrinsic pathway and thus the possibility of an inhibitory effect of protein S on the intrinsic tenase (factor IXa/VIIIa) complex could not be investigated.

In 1995, it was reported that on endothelial cells, protein S inhibited factor X activation by the intrinsic tenase complex in the absence of APC in a dose-dependent manner[93]. Protein S has been observed to bind to immobilized factor VIII and this binding could be reversed by addition of free solution-factor VIII. Incubation of protein S with factor IXa revealed that the inhibitory action of protein S was found to be solely via factor VIIIa. Again binding to phospholipid surfaces was a prerequisite for the inhibitory action of protein S and the inhibition of factor Xa-generating activity by protein S became more pronounced at low phospholipid concentrations. The cleaved form of protein S was found to be equally effective as an intrinsic tenase inhibitor and the complex of protein S and C4BP even appeared to be a somewhat more potent factor VIIIa inhibitor than free protein S [93,94]. The basis of the increased inhibitory activity of the protein S/C4BP complex appeared to be the protein S-independent binding of C4BP to factor VIIIa, factor VIII and the factor VIII/von Willebrand factor complex [94]. Judging from binding experiments with various monoclonal antibodies, the binding of C4BP to factor VIII(a) appeared to be mediated by the alpha-chains of C4BP. A monoclonal antibody that inhibited binding of C4BP to factor VIII also abolished intrinsic tenase inhibition by the protein S/C4BP complex. C4BP alone did not have an effect on intrinsic tenase activity [94].

In the mid-1990s, considerable experimental evidence in favor of an APC-independent activity of protein S accumulated. Nevertheless, questions did arise about the nature and relevance of the APC-independent effects of protein S. One potential problem was the fact that although APC-independent activity of protein S was observed in plasma [9,11,92], no assay existed to quantify this activity and thus there was no evidence for a possible clinical relevance of the APC-independent activity. In 1998 however, an assay for the quantification of APC-independent activity in plasma was developed by van Wijnen and coworkers[95]. Antibodies against protein S were used to neutralize protein S activity in plasma and coagulation, triggered with tissue factor, was monitored in the presence of low molecular weight heparin (LMWH). The ratio between the clotting times of plasma without antibodies against protein S (native plasma) and with antibodies against protein S was defined as a measure for the APC-independent activity of protein S (Protein S-dependent Anticoagulant Ratio, PSdAR). Interestingly, the authors found a normal PSdAR in plasma of patients who were taking oral anticoagulants, despite the approximately 50% decreased levels of protein S [95]. The increased APC-independent activity of protein S (relative to normal levels of protein S) observed in patients receiving oral anticoagulants may be attributed to a possible change in protein S as a consequence of the oral anticoagulant treatment. On the other hand, the possibility also exists that the specific plasma environment may have undergone changes during oral anticoagulant therapy which facilitate the APC-independent action of protein S. The assay presented by van Wijnen et al. was sufficiently sensitive for APC-independent effects of protein S and good correlations were found between the PSdAR and protein S levels [95]. The APC-independent activity of protein S in plasma was greatly enhanced in the presence of LMWH and the PSdAR was increased when plasma levels of factor VII and factor X became lower than 50%. To circumvent possible perturbing effects of low levels of coagulation factors on the PSdAR, sample plasmas were mixed 1:1 with protein S-depleted plasma, thereby achieving good correlations between PSdAR and protein S levels.

This approach, though elegant and useful for the creation of a robust assay may, along with the addition of LMWH, both introduce and mask specific properties of the sample plasma and may potentially lead to incorrect assessment of the actual APC-independent activity of protein S.

A second curious aspect of the APC-independent activity of protein S was the reported difference in the extent of APC-independent activity between various protein S preparations which has been observed in several occasions [10,91,96], indicating that perhaps only a sub-fraction of protein S molecules may be active as APC-independent inhibitor. In an effort to explain the different APC-independent activities of different protein S preparations, van Wijnen and coworkers compared protein S purified from four different sources [96]. Large differences in phospholipid binding properties were found between the protein S preparations and the extent of the APC-independent inhibition of both prothrombinase and intrinsic tenase was observed to correlate with the affinity for negatively charged phospholipids [96].

Again, binding to phospholipid vesicles was found to be essential for the expression of APC-independent activity. In fact, later it has been proposed that competition for binding sites on phospholipid surfaces was the only mechanism by which protein S inhibited thrombin generation in the absence of APC [97]. The APC-independent activity of protein S was pronounced at limiting concentrations of phospholipids and on quiescent platelets but no APC-independent effect of protein S was observed when high concentrations of phospholipids were present [97,98]. Moreover, the extent of inhibition of prothrombinase by protein S could be predicted by a mathematical model in which only the affinity of protein S for phospholipids was taken into account [97].

Although the mechanism of the APC-independent activity of protein S was brought down to simple competition for phospholipid binding sites, it must be noted that exact characterization of the phospholipid binding properties of protein S itself has been proven problematic. Apart from the wide range of affinities for phospholipids that have been reported for protein S [9,96,97,99,100], peculiar phospholipid binding behavior has also been observed.

Binding of protein S to negatively charged phospholipids has been observed to be effectively inhibited by high concentrations of calcium ions [101] and moreover, human protein S has been shown to exhibit non ideal biphasic binding behavior to phospholipid vesicles and this was attributed to aggregation of protein S and stacking of multiple layers of human protein S on the vesicles [102].

In an effort to explain the reported differences in the extent of APC-independent activity between different protein S preparations, Seré et al. compared two protein S preparations with approximately fourfold difference in APC-independent activity [103]. Both preparations displayed biphasic binding behavior towards negatively charged phospholipids; a rapid initial binding phase was followed by a slower absorption phase. Native polyacrylamide gel analysis and size exclusion chromatography revealed the presence of multimeric forms of protein S in the purified preparations. These multimeric forms of protein S, which were estimated to constitute approximately 5% of total purified protein S, possessed high affinity for negatively charged phospholipids (Kd <1 nM) and appeared to be responsible for the slow association with the phospholipid surface. The remaining fraction of protein S had much less affinity for phospholipids (Kd = 250 nM) and showed rapid association because of its abundance. Separation of purified protein S in a multimeric and a monomeric protein S fraction revealed that the multimeric fraction was 100-fold more active as prothrombinase inhibitor than the monomeric fraction. The more active protein S preparation contained more multimeric protein S and this explained the higher activity of this protein S preparation. When multimeric protein S was removed from both preparations, the difference between these preparations disappeared. These data suggested that the multimeric forms of protein S were largely responsible for the APC-independent activity of purified protein S and that the differences in both phospholipid binding and APC-independent activity between various protein S preparations were due to differences in the amounts of multimers present in these preparations. Multimeric forms of protein S were not detected in plasma [103].

The apparent absence of protein S multimers in plasma raises the question about the origin of the multimeric forms of protein S. A possible explanation for the presence of multimers in purified protein S may be self-association during purification. For example, self-association of protein S has previously been observed in the presence of EDTA [104], a reagent commonly used in protein purifications. Although the existence of protein S multimers may not have any physiologic relevance, some previously reported characteristics of the APC-independent activity of protein S might be a subject for reconsideration. One of these characteristics may be the reported strong dependence of phospholipids [96,97] which may solely be ascribed to the phospholipid-dependent action of the protein S multimers. Also, several observations of APC-independent activity in plasma were performed by adding purified protein S to protein S-depleted plasma [9,11,96] and these purified preparations may have contained the multimeric form of protein S as well. In addition, in the only two reports that describe "endogenous" APC-independent activity of protein S in plasma, considerable amounts of heparin were added to plasma in order to measure APC-independent effects of protein S [92,95]. Therefore, this may give rise to questions about the importance of the APC-independent activity of protein S in plasma and procured the need for a plasma assay for the APC-independent activity of protein S in native, untreated plasma.

Clinical and epidemiological aspects of protein S-deficiency

Deficiency of protein S was identified as a risk factor for venous thrombosis quite soon after the description of its APC-cofactor function and many studies investigating protein S as a risk factor for venous thrombosis have been published afterwards. The relative risk for venous thrombosis associated with protein S-deficiency has been found to be 5-11.5[105-107]. Protein S-deficiency is classified into three main categories. In type I protein S-deficiency total protein S levels are reduced, while type III protein S-deficiency is characterized by decreased levels of free protein S.

Type II protein S-deficiency is a qualitative deficiency of protein S which is characterized by impaired function of protein S at normal antigen levels. The quantitative (type I and III) deficiencies of protein S are the most abundant (95%, reviewed by Rezende et al.[108]). It has been proposed that type I and III protein S-deficiencies are different phenotypic manifestations of the same underlying genetic defect[109]. Despite the attention given to protein S-deficiency, the question whether protein S-deficiency is a risk factor for venous thrombosis is still a matter of controversy. The severe phenotype of homozygous protein S-deficiency strongly argues for protein S as an important anticoagulant but it has been surprisingly difficult to establish a clear connection between protein S deficiency and venous (and possibly arterial) thrombosis.

Other functions of protein S

Several studies have indicated that protein S may be involved in other physiologic processes besides the regulation of thrombin formation. Protein S enhances the binding of C4BP to neutrophils[116]. In addition, protein S and protein S/C4BP have been found to bind to apoptotic cells[117] and recently, protein S was shown to be the factor in serum that enhances uptake of apoptotic cells by macrophages[14]. C4BP plays a modulatory role in this enhancement[118].

The observations that protein S expression is inducible by IL-4 in T-cells[28] and that protein S can be cleaved by neutrophil elastase[119] also point towards a role of protein S in the regulation of apoptosis- and inflammation-associated processes. A recent report by Liu and coworkers[13] showed that protein S protects against neuronal damage in an ischemia/reperfusion model. In the future, more direct evidence will most likely be found demonstrating a role of protein S in the regulation of inflammation, more or less independent of its role in the regulation of thrombin formation.

References

1. Di Scipio RG, Hermodson MA, Yates SG, Davie EW. A comparison of human prothrombin, factor IX (Christmas factor), factor X (Stuart factor), and protein S. Biochemistry. 1977;16:698-706
2. Walker FJ. Regulation of activated protein C by a new protein. A possible function for bovine protein S. J Biol Chem. 1980;255:5521-5524
3. Dahlbäck B, Stenflo J. High molecular weight complex in human plasma between vitamin K-dependent protein S and complement component C4b-binding protein. Proc Natl Acad Sci U S A. 1981;78:2512-2516
4. Comp PC, Esmon CT. Recurrent venous thromboembolism in patients with a partial deficiency of protein S. N Engl J Med. 1984;311:1525-1528
5. Comp PC, Nixon RR, Cooper MR, Esmon CT. Familial protein S deficiency is associated with recurrent thrombosis. J Clin Invest. 1984;74:2082-2088
6. Schwarz H, Fischer M, Hopmeier P, Batard M, Griffin J. Plasma protein S deficiency in familial thrombotic disease. Blood. 1984;64:1297-1300.
7. Walker FJ, Chavin SI, Fay PJ. Inactivation of factor VIII by activated protein C and protein S. Arch Biochem Biophys. 1987;252:322-328
8. Koedam JA, Meijers JC, Sixma JJ, Bouma BN. Inactivation of human factor VIII by activated protein C. Cofactor activity of protein S and protective effect of von Willebrand factor. J Clin Invest. 1988;82:1236-1243
9. Hackeng TM, van 't Veer C, Meijers JC, Bouma BN. Human protein S inhibits prothrombinase complex activity on endothelial cells and *platelets via direct interactions with factors Va and Xa. J Biol Chem.* 1994;269:21051-21058
10. Heeb MJ, Mesters RM, Tans G, Rosing J, Griffin JH. Binding of protein S to factor Va associated with inhibition of prothrombinase that is independent of activated protein C. J Biol Chem. 1993;268:2872-2877
11. Heeb MJ, Rosing J, Bakker HM, Fernandez JA, Tans G, Griffin JH. Protein S binds to and inhibits factor Xa. Proc Natl Acad Sci U S A. 1994;91:2728-2732
12. Stitt TN, Conn G, Gore M, Lai C, Bruno J, Radziejewski C, Mattsson K, Fisher J, Gies DR, Jones PF, et al. The anticoagulation factor protein S and its relative, Gas6, are ligands for the Tyro 3/Axl family of receptor tyrosine kinases. Cell. 1995;80:661-670
13. Liu D, Guo H, Griffin JH, Fernandez JA, Zlokovic BV. Protein S confers neuronal protection during ischemic/hypoxic injury in mice. Circulation. 2003;107:1791-1796

14. Anderson HA, Maylock CA, Williams JA, Paweletz CP, Shu H, Shacter E. Serum-derived protein S binds to phosphatidylserine and stimulates the phagocytosis of apoptotic cells. Nat Immunol. 2003;4:87-91
15. Ploos van Amstel JK, van der Zanden AL, Bakker E, Reitsma PH, Bertina RM. Two genes homologous with human protein S cDNA are located on chromosome 3. Thromb Haemost. 1987;58:982-987
16. Schmidel DK, Tatro AV, Phelps LG, Tomczak JA, Long GL. Organization of the human protein S genes. Biochemistry. 1990;29:7845-7852
17. Watkins PC, Eddy R, Fukushima Y, Byers MG, Cohen EH, Dackowski WR, Wydro RM, Shows TB. The gene for protein S maps near the centromere of human chromosome 3. Blood. 1988;71:238-241
18. Hoskins J, Norman DK, Beckmann RJ, Long GL. Cloning and characterization of human liver cDNA encoding a protein S precursor. Proc Natl Acad Sci U S A. 1987;84:349-353
19. He X, Shen L, Bjartell A, Dahlbäck B. The gene encoding vitamin K-dependent anticoagulant protein S is expressed in multiple rabbit organs as demonstrated by northern blotting, in situ hybridization, and immunohistochemistry. J Histochem Cytochem. 1995;43:85-96
20. Shimokawa T, Yamamoto K, Yamafuji E, Kojima T, Saito H. Expression of protein S in the murine heart and cultured mouse cardiomyocytes is down-regulated by cytokines. Thromb Haemost. 2001;86:623-629
21. Fair DS, Marlar RA, Levin EG. Human endothelial cells synthesize protein S. Blood. 1986;67:1168-1171
22. Hooper WC, Phillips DJ, Evatt BL. Endothelial cell protein S synthesis is upregulated by the complex of IL-6 and soluble IL-6 receptor. Thromb Haemost. 1997;77:1014-1019
23. Stern D, Brett J, Harris K, Nawroth P. Participation of endothelial cells in the protein C-protein S anticoagulant pathway: the synthesis and release of protein S. J Cell Biol. 1986;102:1971-1978
24. Maillard C, Berruyer M, Serre CM, Dechavanne M, Delmas PD. Protein-S, a vitamin K-dependent protein, is a bone matrix component synthesized and secreted by osteoblasts. Endocrinology. 1992;130:1599-1604
25. Malm J, He XH, Bjartell A, Shen L, Abrahamsson PA, Dahlbäck B. Vitamin K-dependent protein S in Leydig cells of human testis. Biochem J. 1994;302 (Pt 3):845-850
26. Schwarz HP, Heeb MJ, Wencel-Drake JD, Griffin JH. Identification and quantitation of protein S in human platelets. Blood. 1985;66:1452-1455
27. Schwarz HP, Heeb MJ, Lammle B, Berrettini M, Griffin JH. Quantitative immunoblotting of plasma and platelet protein S. Thromb Haemost. 1986;56:382-386
28. Smiley ST, Boyer SN, Heeb MJ, Griffin JH, Grusby MJ. Protein S is inducible by interleukin 4 in T cells and inhibits lymphoid cell procoagulant activity. Proc Natl Acad Sci U S A. 1997;94:11484-11489

29. Edenbrandt CM, Lundwall A, Wydro R, Stenflo J. Molecular analysis of the gene for vitamin K dependent protein S and its pseudogene. Cloning and partial gene organization. Biochemistry. 1990;29:7861-7868
30. Lundwall A, Dackowski W, Cohen E, Shaffer M, Mahr A, Dahlback B, Stenflo J, Wydro R. Isolation and sequence of the cDNA for human protein S, a regulator of blood coagulation. Proc Natl Acad Sci U S A. 1986;83:6716-6720
31. Fujita T, Gigli I, Nussenzweig V. Human C4-binding protein. II. Role in proteolysis of C4b by C3b-inactivator. J Exp Med. 1978;148:1044-1051
32. Hillarp A, Dahlbäck B. Novel subunit in C4b-binding protein required for protein S binding. J Biol Chem. 1988;263:12759-12764
33. Arenzana N, Rodriguez de Cordoba S, Rey-Campos J. Expression of the human gene coding for the alpha-chain of C4b-binding protein, C4BPA, is controlled by an HNF1-dependent hepatic-specific promoter. Biochem J. 1995;308 (Pt 2):613-621
34. Criado-Garcia O, Fernaud-Espinosa I, Bovolenta P, Sainz de la Cuesta R, Rodriguez de Cordoba S. Expression of the beta-chain of the complement regulator C4b-binding protein in human ovary. Eur J Cell Biol. 1999;78:657-664
35. Hardig Y, Dahlback B. The amino-terminal module of the C4b-binding protein beta-chain contains the protein S-binding site. J Biol Chem. 1996;271:20861-20867
36. Hardig Y, Hillarp A, Dahlback B. The amino-terminal module of the C4b-binding protein alpha-chain is crucial for C4b binding and factor I-cofactor function. Biochem J. 1997;323 (Pt 2):469-475
37. Perez-Caballero D, Alberti S, Vivanco F, Sanchez-Corral P, Rodriguez de Cordoba S. Assessment of the interaction of human complement regulatory proteins with group A Streptococcus. Identification of a high-affinity group A Streptococcus binding site in FHL-1. Eur J Immunol. 2000;30:1243-1253
38. Accardo P, Sanchez-Corral P, Criado O, Garcia E, Rodriguez de Cordoba S. Binding of human complement component C4b-binding protein (C4BP) to Streptococcus pyogenes involves the C4b-binding site. J Immunol. 1996;157:4935-4939
39. Brodeur SR, Angelini F, Bacharier LB, Blom AM, Mizoguchi E, Fujiwara H, Plebani A, Notarangelo LD, Dahlback B, Tsitsikov E, Geha RS. C4b-binding protein (C4BP) activates B cells through the CD40 receptor. Immunity. 2003;18:837-848
40. Webb JH, Villoutreix BO, Dahlbäck B, Blom AM. Localization of a hydrophobic binding site for anticoagulant protein S on the beta -chain of complement regulator C4b-binding protein. J Biol Chem. 2001;276:4330-4337
41. van de Poel RH, Meijers JC, Dahlbck B, Bouma BN. C4b-binding protein (C4BP) beta-chain Short Consensus Repeat-2 specifically contributes to the interaction of C4BP with protein S. Blood Cells Mol Dis. 1999;25:279-286

42. Walker FJ. Properties of chemically modified protein S: effect of the conversion of gamma-carboxyglutamic acid to gamma-methyleneglutamic acid on functional properties. Biochemistry. 1986;25:6305-6311
43. Borowski M, Furie BC, Bauminger S, Furie B. Prothrombin requires two sequential metal-dependent conformational transitions to bind phospholipid. Conformation-specific antibodies directed against the phospholipid-binding site on prothrombin. J Biol Chem. 1986;261:14969-14975
44. Soriano-Garcia M, Padmanabhan K, de Vos AM, Tulinsky A. The Ca2+ ion and membrane binding structure of the Gla domain of Ca-prothrombin fragment 1. Biochemistry. 1992;31:2554-2566
45. Grinnell B, Walls J, Marks C, Glasebrook A, Berg D, Yan S, Bang N. Gamma-carboxylated isoforms of recombinant human protein S with different biologic properties. Blood. 1990;76:2546-2554.
46. Rezende SM, Lane DA, Mille-Baker B, Samama MM, Conard J, Simmonds RE. Protein S Gla-domain mutations causing impaired Ca(2+)-induced phospholipid binding and severe functional protein S deficiency. Blood. 2002;100:2812-2819
47. Meijer-Huizinga F, Mertens K, van Mourik JA. Isolation and characterization of single-chain protein S. Thromb Haemost. 1994;72:408-414
48. Chang GT, Aaldering L, Hackeng TM, Reitsma PH, Bertina RM, Bouma BN. Construction and characterization of thrombin-resistant variants of recombinant human protein S. Thromb Haemost. 1994;72:693-697
49. Long GL, Lu D, Xie RL, Kalafatis M. Human protein S cleavage and inactivation by coagulation factor Xa. J Biol Chem. 1998;273:11521-11526
50. Heeb MJ, Griffin JH. Activated protein C-dependent and -independent anticoagulant activities of protein s have different structural requirements. Blood Cells Mol Dis. 2002;29:190-199
51. Mitchell CA, Hau L, Salem HH. Control of thrombin mediated cleavage of protein S. Thromb Haemost. 1986;56:151-154
52. Suzuki K, Nishioka J, Hashimoto S. Regulation of activated protein C by thrombin-modified protein S. J Biochem (Tokyo). 1983;94:699-705
53. Morboeuf O, Borgel D, Gaussem P, Vincenot A, Pittet JL, Aiach M, Gandrille S. Characterization of cleaved plasma protein S with a monoclonal antibody-based assay. Thromb Haemost. 2000;84:604-610
54. Heeb MJ, Mosher D, Griffin JH. Activation and complexation of protein C and cleavage and decrease of protein S in plasma of patients with intravascular coagulation. Blood. 1989;73:455-461
55. Stenflo J, Lundwall A, Dahlback B. beta-Hydroxyasparagine in domains homologous to the epidermal growth factor precursor in vitamin K-dependent protein S. Proc Natl Acad Sci U S A. 1987;84:368-372

56. Dahlbäck B, Hildebrand B, Linse S. Novel type of very high affinity calcium-binding sites in beta-hydroxyasparagine-containing epidermal growth factor-like domains in vitamin K-dependent protein S. J Biol Chem. 1990;265:18481-18489
57. Stenberg Y, Linse S, Drakenberg T, Stenflo J. The high affinity calcium-binding sites in the epidermal growth factor module region of vitamin K-dependent protein S. J Biol Chem. 1997;272:23255-23260
58. Stenberg Y, Julenius K, Dahlqvist I, Drakenberg T, Stenflo J. Calcium-binding properties of the third and fourth epidermal-growth-factor-like modules in vitamin-K-dependent protein S. Eur J Biochem. 1997;248:163-170
59. Stenberg Y, Muranyi A, Steen C, Thulin E, Drakenberg T, Stenflo J. EGF-like module pair 3-4 in vitamin K-dependent protein S: modulation of calcium affinity of module 4 by module 3, and interaction with factor X. J Mol Biol. 1999;293:653-665
60. Leroy-Matheron C, Gouault-Heilmann M, Aiach M, Gandrille S. A mutation of the active protein S gene leading to an EGF1-lacking protein in a family with qualitative (type II) deficiency. Blood. 1998;91:4608-4615
61. Stenberg Y, Drakenberg T, Dahlback B, Stenflo J. Characterization of recombinant epidermal growth factor (EGF)-like modules from vitamin-K-dependent protein S expressed in Spodoptera cells--the cofactor activity depends on the N-terminal EGF module in human protein S. Eur J Biochem. 1998;251:558-564
62. Hackeng TM, Dawson PE, Kent SB, Griffin JH. Chemical synthesis of human protein S thrombin-sensitive module and first epidermal growth factor module. Biopolymers. 1998;46:53-63
63. Hayashi T, Nishioka J, Shigekiyo T, Saito S, Suzuki K. Protein S Tokushima: abnormal molecule with a substitution of Glu for Lys-155 in the second epidermal growth factor-like domain of protein S. Blood. 1994;83:683-690
64. Hayashi T, Nishioka J, Suzuki K. Molecular mechanism of the dysfunction of protein S(Tokushima) (Lys155-->Glu) for the regulation of the blood coagulation system. Biochim Biophys Acta. 1995;1272:159-167
65. Mille-Baker B, Rezende SM, Simmonds RE, Mason PJ, Lane DA, Laffan MA. Deletion or replacement of the second EGF-like domain of protein S results in loss of APC cofactor activity. Blood. 2003;101:1416-1418
66. Joseph DR. Structure, function, and regulation of androgen-binding protein/sex hormone-binding globulin. Vitam Horm. 1994;49:197-280
67. Joseph D, Baker M. Sex hormone-binding globulin, androgen-binding protein, and vitamin K- dependent protein S are homologous to laminin A, merosin, and Drosophila crumbs protein. FASEB J. 1992;6:2477-2481
68. Beckmann G, Hanke J, Bork P, Reich JG. Merging extracellular domains: fold prediction for laminin G-like and amino-terminal

thrombospondin-like modules based on homology to pentraxins. J Mol Biol. 1998;275:725-730
69. Tisi D, Talts JF, Timpl R, Hohenester E. Structure of the C-terminal laminin G-like domain pair of the laminin alpha2 chain harbouring binding sites for alpha-dystroglycan and heparin. Embo J. 2000;19:1432-1440
70. Grishkovskaya I, Avvakumov GV, Sklenar G, Dales D, Hammond GL, Muller YA. Crystal structure of human sex hormone-binding globulin: steroid transport by a laminin G-like domain. Embo J. 2000;19:504-512
71. Villoutreix BO, Dahlbäck B, Borgel D, Gandrille S, Muller YA. Three-dimensional model of the SHBG-like region of anticoagulant protein S: new structure-function insights. Proteins. 2001;43:203-216
72. He X, Shen L, Malmborg A, Smith K, Dahlbäck B, Linse S. Binding site for C4b-binding protein in vitamin K-dependent protein S fully contained in carboxy-terminal laminin-G-type repeats. A study using recombinant factor IX-protein S chimeras and surface plasmon resonance. Biochemistry. 1997;36:3745-3754.
73. Saposnik B, Borgel D, Aiach M, Gandrille S. Functional properties of the sex-hormone-binding globulin (SHBG)-like domain of the anticoagulant protein S. Eur J Biochem. 2003;270:545-555
74. Walker FJ. Characterization of a synthetic peptide that inhibits the interaction between protein S and C4b-binding protein. J Biol Chem. 1989;264:17645-17648
75. Nelson RM, Long GL. Binding of protein S to C4b-binding protein. Mutagenesis of protein S. J Biol Chem. 1992;267:8140-8145
76. Linse S, Hardig Y, Schultz DA, Dahlback B. A region of vitamin K-dependent protein S that binds to C4b binding protein (C4BP) identified using bacteriophage peptide display libraries. J Biol Chem. 1997;272:14658-14665
77. Fernandez JA, Heeb MJ, Griffin JH. Identification of residues 413-433 of plasma protein S as essential for binding to C4b-binding protein. J Biol Chem. 1993;268:16788-16794
78. Fernandez JA, Griffin JH, Chang GT, Stam J, Reitsma PH, Bertina RM, Bouma BN. Involvement of amino acid residues 423-429 of human protein S in binding to C4b-binding protein. Blood Cells Mol Dis. 1998;24:101-112; discussion 113
79. Giri TK, Linse S, Garcia de Frutos P, Yamazaki T, Villoutreix BO, Dahlback B. Structural requirements of anticoagulant protein S for its binding to the complement regulator C4b-binding protein. J Biol Chem. 2002;277:15099-15106
80. Evenas P, Garcia De Frutos P, Linse S, Dahlbäck B. Both G-type domains of protein S are required for the high-affinity interaction with C4b-binding protein. Eur J Biochem. 1999;266:935-942
81. Dahlbäck B, Frohm B, Nelsestuen G. High affinity interaction between C4b-binding protein and vitamin K-dependent protein S in the presence of calcium. Suggestion of a third component in blood regulating the interaction. J Biol Chem. 1990;265:16082-16087

82. Heeb MJ, Kojima Y, Rosing J, Tans G, Griffin JH. C-terminal residues 621-635 of protein S are essential for binding to factor Va. J Biol Chem. 1999;274:36187-36192
83. Nyberg P, He X, Hardig Y, Dahlbäck B, Garcia de Frutos P. Stimulation of Sky tyrosine phosphorylation by bovine protein S--domains involved in the receptor-ligand interaction. Eur J Biochem. 1997;246:147-154
84. Evenas P, Dahlbäck B, Garcia de Frutos P. The first laminin G-type domain in the SHBG-like region of protein S contains residues essential for activation of the receptor tyrosine kinase sky. Biol Chem. 2000;381:199-209
85. Godowski PJ, Mark MR, Chen J, Sadick MD, Raab H, Hammonds RG. Reevaluation of the roles of protein S and Gas6 as ligands for the receptor tyrosine kinase Rse/Tyro 3. Cell. 1995;82:355-358
86. Walker FJ. Regulation of activated protein C by protein S. The role of phospholipid in factor Va inactivation. J Biol Chem. 1981;256:11128-11131
87. Yegneswaran S, Wood GM, Esmon CT, Johnson AE. Protein S alters the active site location of activated protein C above the membrane surface. A fluorescence resonance energy transfer study of topography. J Biol Chem. 1997;272:25013-25021
88. Hackeng TM, Yegneswaran S, Johnson AE, Griffin JH. Conformational changes in activated protein C caused by binding of the first epidermal growth factor-like module of protein S. Biochem J. 2000;349 Pt 3:757-764
89. Smirnov MD, Safa O, Regan L, Mather T, Stearns-Kurosawa DJ, Kurosawa S, Rezaie AR, Esmon NL, Esmon CT. A chimeric protein C containing the prothrombin Gla domain exhibits increased anticoagulant activity and altered phospholipid specificity. J Biol Chem. 1998;273:9031-9040
90. Yegneswaran S, Smirnov MD, Safa O, Esmon NL, Esmon CT, Johnson AE. Relocating the active site of activated protein C eliminates the need for its protein S cofactor. A fluorescence resonance energy transfer study. J Biol Chem. 1999;274:5462-5468
91. Mitchell C, Kelemen S, Salem H. The anticoagulant properties of a modified form of protein S. Thromb Haemost. 1988;60:298-304.
92. van 't Veer C, Hackeng TM, Biesbroeck D, Sixma JJ, Bouma BN. Increased prothrombin activation in protein S-deficient plasma under flow conditions on endothelial cell matrix: an independent anticoagulant function of protein S in plasma. Blood. 1995;85:1815-1821
93. Koppelman SJ, Hackeng TM, Sixma JJ, Bouma BN. Inhibition of the intrinsic factor X activating complex by protein S: evidence for a specific binding of protein S to factor VIII. Blood. 1995;86:1062-1071
94. Koppelman SJ, van't Veer C, Sixma JJ, Bouma BN. Synergistic inhibition of the intrinsic factor X activation by protein S and C4b-binding protein. Blood. 1995;86:2653-2660

95. van Wijnen M, van 't Veer C, Meijers JC, Bertina RM, Bouma BN. A plasma coagulation assay for an activated protein C-independent anticoagulant activity of protein S. Thromb Haemost. 1998;80:930-935
96. van Wijnen M, Stam JG, van't Veer C, Meijers JC, Reitsma PH, Bertina RM, Bouma BN. The interaction of protein S with the phospholipid surface is essential for the activated protein C-independent activity of protein S. Thromb Haemost. 1996;76:397-403
97. van 't Veer C, Butenas S, Golden N, Mann K. Regulation of prothrombinase activity by protein S. Thromb Haemost. 1999;82:80-87.
98. van 't Veer C, Kalafatis M, Bertina RM, Simioni P, Mann KG. Increased tissue factor-initiated prothrombin activation as a result of the Arg506 --> Gln mutation in factor VLEIDEN. J Biol Chem. 1997;272:20721-20729
99. Dahlbäck B, Wiedmer T, Sims PJ. Binding of anticoagulant vitamin K-dependent protein S to platelet-derived microparticles. Biochemistry. 1992;31:12769-12777
100. Hackeng TM, Hessing M, van 't Veer C, Meijer-Huizinga F, Meijers JC, de Groot PG, van Mourik JA, Bouma BN. Protein S binding to human endothelial cells is required for expression of cofactor activity for activated protein C. J Biol Chem. 1993;268:3993-4000
101. Schwalbe RA, Ryan J, Stern DM, Kisiel W, Dahlbäck B, Nelsestuen GL. Protein structural requirements and properties of membrane binding by gamma-carboxyglutamic acid-containing plasma proteins and peptides. J Biol Chem. 1989;264:20288-20296
102. Schwalbe R, Dahlbäck B, Hillarp A, Nelsestuen G. Assembly of protein S and C4b-binding protein on membranes. J Biol Chem. 1990;265:16074-16081
103. Sere KM, Janssen MP, Willems GM, Tans G, Rosing J, Hackeng TM. Purified protein S contains multimeric forms with increased APC-independent anticoagulant activity. Biochemistry. 2001;40:8852-8860
104. Pauls JE, Hockin MF, Long GL, Mann KG. Self-association of human protein S. Biochemistry. 2000;39:5468-5473
105. Simmonds RE, Ireland H, Lane DA, Zoller B, Garcia de Frutos P, Dahlbäck B. Clarification of the risk for venous thrombosis associated with hereditary protein S deficiency by investigation of a large kindred with a characterized gene defect. Ann Intern Med. 1998;128:8-14
106. Makris M, Leach M, Beauchamp NJ, Daly ME, Cooper PC, Hampton KK, Bayliss P, Peake IR, Miller GJ, Preston FE. Genetic analysis, phenotypic diagnosis, and risk of venous thrombosis in families with inherited deficiencies of protein S. Blood. 2000;95:1935-1941
107. Martinelli I, Mannucci PM, De Stefano V, Taioli E, Rossi V, Crosti F, Paciaroni K, Leone G, Faioni EM. Different risks of thrombosis in four coagulation defects associated with inherited thrombophilia: a study of 150 families. Blood. 1998;92:2353-2358
108. Rezende SM, Simmonds RE, Lane DA. Coagulation, inflammation, and apoptosis: different roles for protein S and the protein S-C4b binding protein complex. Blood. 2004;103:1192-1201

109. Zoller B, Garcia de Frutos P, Dahlbäck B. Evaluation of the relationship between protein S and C4b-binding protein isoforms in hereditary protein S deficiency demonstrating type I and type III deficiencies to be phenotypic variants of the same genetic disease. Blood. 1995;85:3524-3531
110. Faioni E, Valsecchi C, Palla A, Taioli E, Razzari C, Mannucci P. Free protein S deficiency is a risk factor for venous thrombosis. Thromb Haemost. 1997;78:1343-1346.
111. Goodwin AJ, Rosendaal FR, Kottke-Marchant K, Bovill EG. A review of the technical, diagnostic, and epidemiologic considerations for protein S assays. Arch Pathol Lab Med. 2002;126:1349-1366
112. Persson KE, Hillarp A, Dahlbäck B. Analytical considerations for free protein S assays in protein S deficiency. Thromb Haemost. 2001;86:1144-1147
113. Boerger LM, Morris PC, Thurnau GR, Esmon CT, Comp PC. Oral contraceptives and gender affect protein S status. Blood. 1987;69:692-694
114. Henkens CM, Bom VJ, Van der Schaaf W, Pelsma PM, Sibinga CT, de Kam PJ, van der Meer J. Plasma levels of protein S, protein C, and factor X: effects of sex, hormonal state and age. Thromb Haemost. 1995;74:1271-1275
115. Liberti G, Bertina R, Rosendaal F. Hormonal state rather than age influences cut-off values of protein S: reevaluation of the thrombotic risk associated with protein S deficiency. Thromb Haemost. 1999;82:1093-1096.
116. Furmaniak-Kazmierczak E, Hu CY, Esmon CT. Protein S enhances C4b binding protein interaction with neutrophils. Blood. 1993;81:405-411
117. Webb JH, Blom AM, Dahlback B. Vitamin K-dependent protein S localizing complement regulator C4b-binding protein to the surface of apoptotic cells. J Immunol. 2002;169:2580-2586
118. Kask L, Trouw LA, Dahlback B, Blom AM. The C4b-binding protein-protein S complex inhibits the phagocytosis of apoptotic cells. J Biol Chem. 2004
119. Oates AM, Salem HH. The binding and regulation of protein S by neutrophils. Blood Coagul Fibrinolysis. 1991;2:601-607

Chapter 3

The Ser460Pro mutation in recombinant protein S Heerlen does not affect its APC-cofactor and APC-independent anticoagulant activities.

Koenen RR, Gomes L, Tans G, Rosing J and Hackeng TM.

Thrombosis and Haemostasis, 2004, 91, 1105-1114.

Abstract

Protein S is a vitamin K-dependent plasma protein that functions as an APC-cofactor, but also exhibits anticoagulant activity in the absence of APC. The Heerlen polymorphism of protein S is characterized by a Ser460Pro substitution and lacks glycosylation at Asn458. It is associated with decreased protein S levels due to selective deficiency of free protein S Heerlen. To understand the lack of thrombotic complications associated with the protein S Heerlen mutation, we compared recombinant protein S Heerlen, wild type (wt) protein S and plasma-derived protein S. wt-Protein S and protein S Heerlen each bound 1:1 to C4BP with dissociation constants of 0.27 and 0.33 nM, respectively. Both wt-protein S and protein S Heerlen, either free or in complex with C4BP, were equally active as prothrombinase inhibitors in the absence of APC. All three protein S preparations stimulated APC-catalyzed inactivation of normal FVa, FVa_{Leiden} and FVIIIa to the same extent. If extrapolated to plasma, it is not likely that the decreased free protein S levels in carriers of the protein S Heerlen mutation are compensated by an increased anticoagulant activity of protein S Heerlen-C4BP complexes. It is possible that an unrecognized plasma factor selectively enhances the anticoagulant activity of protein S Heerlen. If not, the reduction of free protein S levels in heterozygous protein S Heerlen-carriers combined with (low) normal total protein S levels apparently minimally affects the total anticoagulant activity of protein S (APC-cofactor and APC-independent activity) and hence is not associated with increased risk of venous thrombosis.

Introduction

The vitamin K-dependent coagulation factor protein S is a negative regulator of blood coagulation. Its physiological importance has been emphasized by associations of deficiencies in protein S with an increased risk for the development of venous thrombosis [1,2]. Protein S is a 75 kDa post-translationally modified protein that contains three glycosylated asparagines in the C-terminal SHBG-like domain. The SHBG-like domain has been reported to mediate high affinity binding of protein S to the beta-chain of C4BP, a component of the classical complement cascade [3]. Approximately 60% of protein S present in plasma circulates in complex with C4BP [4].

Protein S is a cofactor for activated protein C (APC) in the inactivation of FVIIIa [5,6] and FVa [7,8]. FVa contains three cleavage sites for APC that are located in the heavy chain of the molecule: Arg306, Arg506 and Arg679. Rapid cleavage by APC at Arg506 results in the formation of an intermediate FVa molecule with decreased activity. Cleavage of FVa by APC at Arg306, which is accelerated up to 27-fold by protein S [9], results in complete inactivation of the FVa molecule [8]. Until recently, significant acceleration of the APC-catalyzed cleavage of FVa at R506 by protein S has not been found but recent reports demonstrate that also the acceleration of the cleavage at the FVa Arg506 site by protein S is enhanced in the presence of particular phospholipids [9,10]. APC-mediated cleavage at Arg679 has been considered not to be physiologically important but recent findings indicate that cleavage at this site is also enhanced by protein S and contributes to the inactivation of FVa variants Hong Kong and Cambridge which have mutations at Arg306, rendering this site uncleavable by APC [11].

Protein S also functions as a cofactor for APC in the FVIIIa inactivation [5,6]. The acceleration of APC-mediated FVIIIa inactivation by protein S alone is moderate, but the APC-cofactor function of protein S is enhanced in the presence of FV. In this way, FV and protein S work as synergistic cofactors for APC in the inactivation of FVIIIa [12,13]. However, the synergy between protein S and FV is absent when protein S is in complex with C4BP [14].

During the past decade it has been shown that protein S is able to directly inhibit thrombin generation in the absence of APC [15,16]. Competition for negatively charged phospholipids as well as interactions with FXa and FVa have been reported as mechanisms underlying the APC-independent anticoagulant activity of protein S [15,17-21]. Protein S retains APC-independent anticoagulant activity when in complex with C4BP [16], whereas after binding to C4BP its cofactor activity in FVa inactivation is entirely lost [22].

In the early 1990s, an abnormal variant of protein S was reported and was named protein S Heerlen [23]. Protein S Heerlen contains a Ser to Pro substitution at amino acid position 460, resulting in the loss of the N-glycosylation consensus sequence (Asn-Xxx-Ser) and thus N-linked glycosylation at Asn458. The prevalence of the Ser460Pro mutation among the healthy population was estimated at approximately 0.5% and it was initially considered a neutral polymorphism. Later it was shown however, that the Ser460Pro mutation is associated with quantitative protein S-deficiency showing low free protein S levels [24]. However in one study, the Ser460Pro mutation has been found in families with normal phenotypes, thus weakening the association between this mutation and free protein S-deficiency [25]. More recently, homozygosity for the Ser460Pro mutation was found in a female carrier with low total levels of protein S [26]. Interestingly, this carrier had not yet suffered from thrombotic complications. In the same study, slightly but significantly lower total protein S levels were found in the Ser460Pro carriers, indicating that this mutation may affect overall protein S levels. The Ser460Pro mutation has been found in 22% of protein S deficient (< 65%) patients with venous thrombosis, although in about half of the carriers another risk factor was present [27]. It has been reported that protein S Heerlen displayed normal APC cofactor activity in the inactivation of normal FVa [28] and was a poor cofactor in the inactivation of FVa_{Leiden} leading to the postulation that the FV_{Leiden} and protein S Ser460Pro mutations may have a synergistic thrombophilic effect [29]. However, as yet, no clear correlation between the Ser460Pro mutation and venous thrombosis has been established.

It is still unclear why the plasma concentration of free protein S in Ser460Pro carriers is lower than the concentration of free protein S in normal individuals. One possible reason for the abnormal ratio of free and complexed protein S Heerlen may be an altered stoichiometry of the binding of protein S Heerlen to C4BP. One study reported a 2:1 stoichiometry for protein S Heerlen binding to C4BP [24]. This finding however, was not confirmed by two other studies that reported a 1:1 binding of recombinant protein S that lacks glycosylation at Asn458 to C4BP [30,31]. It was hypothesized that the low free protein S levels in protein S Heerlen carriers might be caused by increased clearance of free, but not complexed protein S Heerlen from plasma [26,32], however, conclusive data are still lacking.

In the present paper we report the results of a study that was aimed to provide a biochemical rationale for the general lack of prothrombotic complications associated with low free protein S levels with the protein S Heerlen mutation. Protein S Heerlen and normal protein S were characterized for their ability to bind to C4BP, for their APC-independent anticoagulant activity and APC-cofactor activity in the inactivation of FVIIIa as well as FVa and FVa_{Leiden}.

Materials and methods

Materials

Protein S, APC and human FXa were purchased from Enzyme Research Laboratories (ERL, South Bend, IN, USA). HEPES, fibronectin and BSA (clinical reagent grade) were from ICN (Costa Mesa, CA, USA). Fetal bovine serum, non-essential amino acids, antibiotic/antimycotic, Dulbecco's modified Eagle's medium with GlutaMAX and 4.5 g/L glucose (DMEM), Optimem 1 and lipofectin were obtained from Gibco-BRL (Gaithersburg, MD, USA). G418 sulfate and the pGEM3Zf(+) plasmid vector were purchased from Promega (Madison, WI, USA). DOPC and DOPS were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Chromogenic substrates S2238 and S2765 were supplied by Chromogenix (Mölndal, Sweden).

Q-Sepharose fast flow and QAE-Sephadex ion-exchange resins were purchased from Pharmacia (Uppsala, Sweden). The CM5-Biosensor chip and amine coupling kit were purchased from Biacore AB (Uppsala, Sweden). Unconjugated and horseradish peroxidase (HRP) conjugated rabbit polyclonal anti-protein S antibodies were obtained from DAKO (Glostrup, Denmark). Benzamidine and poly-L-lysine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine FIXa and FX were kind gifts from Dr. S. Rósen, Chromogenix Sweden. Proprietary phospholipids PL-1 were a kind gift from K. van Assche, Instrumentation Laboratory, Breda Netherlands. Plasma immunopurified FVIII (Monoclate) was purchased from Aventis Behring (Kankakee, IL, USA). Other reagents were of the highest grade available. Bovine FXa, human prothrombin, thrombin and total FV were purified as previously described [33].

Site directed mutagenesis

The Ser460Pro mutation in protein S was introduced using the PCR-based "megaprimer" method [34] with the cDNA coding for protein S (kindly provided by Dr. B.N. Bouma) as the template essentially the same as described [28,29]. cDNAs coding for protein S WT and Heerlen were cloned into pcDNA3 (Invitrogen, San Diego, CA, USA). The presence of the Ser460Pro mutation and the absence of errors in the cDNAs were confirmed by PCR-based DNA sequencing on the ABI 310 automated sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) using fluorescent dye terminators.

Stable transfection of HEK293 cells

HEK293 cells, cultured in poly-L-Lysine coated T75 flasks (Nunc, Roskilde, Denmark) in DMEM supplemented with 10% fetal bovine serum, 1x non-essential amino acids and 1x antibiotic/antimycotic were transfected with pcDNA3 containing either wild type or mutant protein S cDNA, using Lipofectin as the transfection reagent. Forty-eight hours after the transfection the contents of each T25 flask were split into two square (120x120 mm) poly-L-lysine coated Petri-dishes (Greiner, Kremsmünster, Austria).

Colonies were grown for three weeks in complete DMEM medium supplemented with 600 µg/mL G418 sulphate. The best producing protein S wild type and Heerlen HEK293 clones were grown to confluence in fibronectin-coated T175 flasks (Nunc) and the serum-containing growth medium was replaced with Optimem 1 with GlutaMAX supplemented with 10 µg/mL vitamin K (Konakion, Roche), 100 mg/mL $CaCl_2$, 1x antibiotic/antimycotic and 200 µg/mL G418 sulphate. Medium was drawn from the cells and the conditioned media were stored in the presence of 5 mM benzamidine at –80 °C until further processing.

Purification of recombinant protein S.

wt-protein S and protein S Heerlen were purified by ion exchange chromatography. The conditioned media were adjusted to 5 mM EDTA and loaded onto a Q-Sepharose fast flow ion exchange column pre-equilibrated with 25 mM Hepes, 150 mM NaCl (pH 7.4). After loading, the column was extensively washed with 25 mM Hepes, 150 mM NaCl (pH 7.4) until OD280 was below 0.007. Protein S was eluted using 20 mM $CaCl_2$ in 25 mM Hepes, 110 mM NaCl (pH 7.4). Subsequently, remaining protein was removed in two steps using 25 mM Hepes, 500 mM NaCl (pH 7.4) and 25 mM Hepes, 1 M NaCl (pH 7.4). The protein S containing fractions were adjusted with EDTA to 25 mM and reloaded on the column and re-eluted with 25 mM Hepes, 500 mM NaCl (pH 7.4). After extensive dialysis in 25 mM Hepes, 175 mM NaCl (pH 7.7) (Hepes buffered saline, HBS), the concentrations of protein S in the pooled protein S preparations were determined by RP-HPLC analysis. The purity of the preparations was > 90% as observed on SDS PAGE and HPLC. The Gla-content of wt-protein S and protein S Heerlen was determined by VITAK BV (Maastricht, the Netherlands).

Determination of protein S concentration using RP-HPLC

Because immunological differences of protein S Heerlen could potentially lead to incorrect concentration determination in antibody-based assays (e.g. ELISA), concentrations of recombinant protein S preparations were determined using reverse phase HPLC using protein S (ERL) as a standard. Fixed volumes (25 µL) of plasma protein S (standard), wt-protein S or protein S Heerlen were injected on a Varian Prostar HPLC unit and separated over a Vydac C4-reversed phase analytical column. Linear gradients of 0-60% acetonitrile in 0.1% trifluoroacetic acid/water in 30 min were used to elute bound protein S. The preparations of protein S eluted as single peaks at retention times of 26 minutes. The area under the peak obtained with 5 µg/25 µL of plasma protein S at 214 nm was used to calculate the concentration of wt-protein S and protein S Heerlen in 25 µL injection sample.

Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF)

Plasma protein S, protein S Heerlen and wt- protein S were concentrated and desalted using reversed phase C3 magnetic beads (MB-HIC3; Bruker Daltonics, Billerica, MA, USA). Protein S samples were mixed 1:1 with a saturated matrix solution of sinapinic acid in 50% acetonitrile/water. Subsequently, for each sample, 20 pmoles of protein S were applied in triplicate to a steel MALDI-TOF target. Spectra were recorded on an Autoflex MALDI TOF mass spectrometer (Bruker Daltonics) in a linear mode and a total of 500 spectra for each of the three samples were accumulated.

Preparation of phospholipid vesicles and liposomes

Phospholipid vesicles were prepared in glass test tubes by evaporating 200 µL of a 10 mM DOPS/DOPC (10/90 or 20/80, M/M) stock solution in chloroform/methanol (1:1) under nitrogen flow. The resulting phospholipid film was suspended in 2 mL of HBS and sonicated on ice to clarity using a probe tip sonicator (20 min sonication at 7.5 µm peak to peak amplitude).

Liposomes were prepared by resuspending a dried 20/80 M/M DOPS/DOPC phospholipid film in HBS and vortexing extensively in the presence of glass beads. The liposomes were centrifuged at 10000 x g and washed with HBS to remove small lipid structures.

APC cofactor activity of protein S in the inactivation of FVa

FV (10 nM) purified from normal plasma or from plasma of a homozygous carrier of FV_{Leiden} was activated for 15 minutes in the presence of 2 nM thrombin in HBS containing 5 mM $CaCl_2$ and 5 mg/mL BSA. Diluted FVa or FVa_{Leiden} (0.4 nM) obtained from the activation mixtures was incubated at 37 °C in HBS containing 0.13 nM APC, 40 µM 10/90 DOPS/DOPC vesicles, 3 mM $CaCl_2$, 5 mg/mL BSA, and varying concentrations of protein S. At fixed time points, aliquots were taken from the inactivation mixture in which the FVa concentration was determined by measuring its FXa-cofactor activity at 37 °C in the presence of 4 nM bovine FXa, 40 µM 10/90 DOPS/DOPC vesicles and 500 nM human prothrombin. After 1 minute, the reaction was stopped in 50 mM Tris-HCl, 175 mM NaCl (pH 7.9) containing 0.5 mg/mL ovalbumin and 20 mM EDTA. The amount of thrombin generated was quantified by measuring amidolytic activity at 405/492 nm using the S2238 chromogenic substrate in an SLT plate reader at 37 °C. The rate constants of cleavage at Arg306 and *Arg506 (k306 and k506 respectively) were determined by fitting* time courses of FVa inactivation to a single or a biphasic exponential equation as described earlier [35].

APC cofactor activity of protein S in the inactivation of FVIIIa

Human FVIII (6 pM) and varying concentrations of plasma-protein S, wt-protein S or protein S Heerlen were preincubated with 30 µM phospholipids (PL-1) in the presence or absence of 1.5 nM human FV and with or without 30 nM human APC at 37 °C in HBS containing 100 mM NaCl and 5 mg/mL BSA. After 5 minutes, a mixture of bovine FIXa (0.1 U/ml), bovine FX (0.6 U/ml), human thrombin (4 nM) and calcium chloride (14 mM) was added, resulting in final concentrations of 2 pM FVIIIa, 10 µM phospholipids (PL-1), 0.03 U/ml FIX, 0.2 U/ml FX, 1.33 nM thrombin, 4.7 mM calcium chloride and when present: 0.5 nM FV, 10 nM APC and 0-40 nM of protein S. The reaction mixture was incubated for 5 more minutes before quenching the reaction with 50 mM Tris-HCl, 175 mM NaCl (pH 7.9) containing 0.5 mg/mL ovalbumin and 20 mM EDTA. This resulted in a tenfold dilution of the reaction mixture. FVIIIa activity was calculated from the amount of FXa generated, determined with the chromogenic substrate S2765 in an SLT plate reader at 37 °C at 405/492 nm. Readings were corrected for an assay blank which contained all constituents of the reaction mixture except protein S and factor VIII.

APC-independent anticoagulant activity of protein S

Prothrombinase complex (FXa/FVa/phospholipids) was preincubated with variable amounts of protein S or protein S/C4BP in HBS containing 3 mM $CaCl_2$ and 5 mg/mL BSA at 37 °C. After 10 minutes, prothrombin was added, resulting in final concentrations of 10 pM FVa, 10 pM FXa, 100 nM phospholipid vesicles (20/80, M/M, DOPS/DOPC) and 500 nM prothrombin. Prothrombin activation was followed by transferring aliquots from the reaction mixture at fixed time points to 50 mM Tris-HCl, 175 mM NaCl (pH 7.9) containing 0.5 mg/mL ovalbumin and 20 mM EDTA. The amount of thrombin present in the diluted aliquots from the reaction mixture was measured in an SLT plate reader at 37 °C and 405/492 nm using the chromogenic substrate S2238. The rate of thrombin formation was taken as a measure of prothrombinase activity.

Purification of beta-chain containing C4BP

The protein S/C4BP complex was isolated from 20 units of freshly frozen plasma (5 L) as previously described [6]. Briefly, the plasma was subjected to barium citrate absorption, ammonium sulphate precipitation and QAE-Sephadex anion exchange chromatography. The protein S/C4BP complex was obtained in an early eluting peak from the QAE-Sephadex column separated from free protein S. C4BP was separated from protein S using RP-HPLC over a Vydac "protein and peptide" C4 semi-prep scale column using a 60 minute, linear gradient of 20-60% acetonitrile in 0.1% trifluoroacetic acid/water at 5 mL/min flow. C4BP and protein S eluted in well-separated peaks with retention times of 35 and 50 minutes, respectively. Fractions (3 mL) were neutralized with 50 µL 1M Tris-HCl (pH 9) immediately upon collection. C4BP-containing fractions were pooled and dialyzed against HBS. The dialyzed C4BP solution was concentrated to 0.9 mg/ml (1.6 µM, measured by OD280 assuming an $\varepsilon^{1\%}_{1cm,\ 280\ nm}$ =14.0 and Mr = 570.000) using a Pall Macrosep spin concentrator (Pall, Port Washington, NY, USA). The purified C4BP was >90 % pure as judged from SDS PAGE analysis, contained no detectable protein S (measured by ELISA) and fully retained protein S binding ability as measured by the Enzyme-Linked Ligandsorbent Assay (ELSA) described by Giri et al. [36].

Surface plasmon resonance analysis

Beta-chain containing C4BP was immobilized on a CM5-biosensor chip at indicated densities using the amine-coupling kit following instructions of the supplier. Routinely, a control channel was activated and blocked using the amine-coupling kit in the absence of protein. Binding to coated channels was corrected for binding to non-coated channels (<1% of binding to coated channels). SPR analysis was performed in 20 mM Hepes-NaOH (pH 7.4), 150 mM NaCl, 3 mM $CaCl_2$ supplemented with 0.05% (v/v) Tween-20 and 0.01% (v/v) NaN_3 at indicated flow-rates. The C4BP sensor chip was regenerated using 100 mM HCl.

Analysis of Quantitative SPR Data

For analysis of association and dissociation curves of the sensorgrams, BiaEvalution software was used (Biacore AB). Interaction constants were determined separately by performing non-linear global fitting of data corrected for bulk refractive index changes. Data were fitted to a one-site binding model. Accuracy of fits were judged from residual plots and statistical parameters employing previously described equations [37].

Removal of multimeric protein S by liposome centrifugation

Multimeric protein S was removed from protein S preparations by liposome extraction essentially as described [21]. Plasma-protein S (2.0 µM), wt-protein S (3.0 µM) and protein S Heerlen (2.0 µM) were incubated under gentle agitation at room temperature with 300 µM liposomes in HBS containing 5 mg/mL BSA and 3 mM $CaCl_2$. After 1.5 hours, the liposomes were separated from the solution by centrifugation at 10000 g in an Eppendorf 5417C centrifuge. The "multimer-free" supernatant was centrifuged for 25 minutes at 110000 g in a Beckman TLA-100 centrifuge in order to remove residual liposomes. The absence of multimeric protein S was confirmed by western blot analysis using HRP conjugated anti-protein S antibodies (data not shown). Protein S concentrations were determined as described above.

Results

Expression, purification and characterization of wt-protein S and protein S Heerlen

HEK 293 cell-culture showed expression levels of approximately 1.1 $\mu g/cm^2$ per day of both wt-protein S and protein S Heerlen with typical purification yields from 1 L of conditioned medium of approximately 1-1.5 mg. Both wt-protein S and protein S Heerlen preparations contained the expected average Gla-amounts of 11.2 and 11.3 respectively, and were thus concluded to be fully gamma-carboxylated. SDS-PAGE analysis showed an increased mobility of protein S Heerlen combined with a slightly more narrow appearance of the protein S Heerlen-band, indicating the lack of glycosylation at Asn458 (Fig. 1A). wt-Protein S co-migrated with a commercial plasma-purified protein S preparation (protein S ERL). MALDI-TOF mass spectral analysis conclusively showed a mass reduction (± 3 kDa) for the protein S Heerlen variant compared with wt-protein S, in agreement with a deletion of a carbohydrate chain from Asn458 (Fig 1B). Concentrations of the recombinant constructs were determined by RP-HPLC as described.

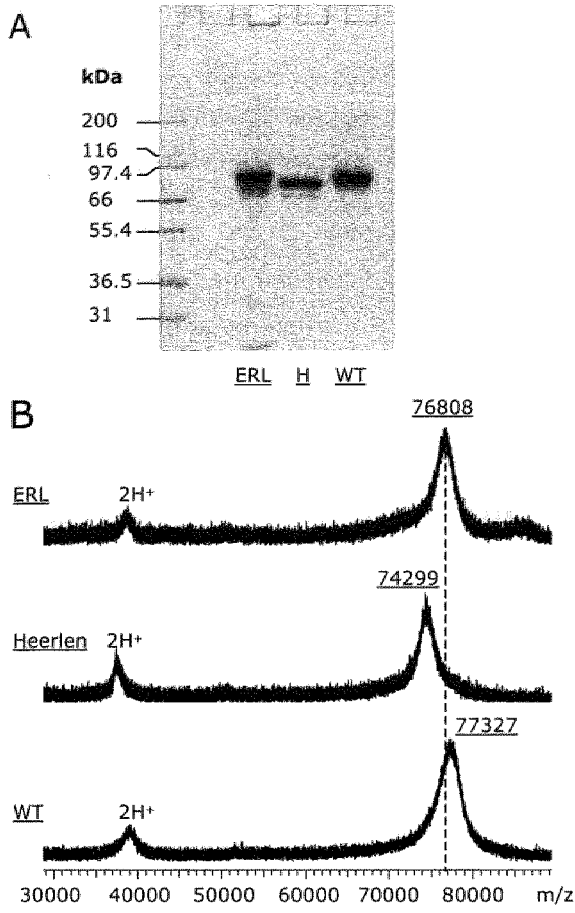

Figure 1: SDS-PAGE and MALDI-TOF analysis of protein S preparations.

A: 2.5 µg of plasma protein S (ERL), protein S Heerlen (H) and wt-protein S (wt) were applied to 10% SDS-polyacrylamide gel electrophoresis under reducing conditions. Molecular weight standards (kDa) are indicated. B: 10 pmoles plasma protein S (ERL), protein S Heerlen (Heerlen) and wt-protein S (wt) were applied in triplicate to a steel MALDI target plate with Sinapinic acid as matrix. From each protein S preparation 500 MALDI-TOF scans in linear mode were accumulated.

Binding of wt-protein S and protein S Heerlen to immobilized C4BP

In order to compare the interaction of wt-protein S and protein S Heerlen with C4BP, binding of these proteins to immobilized C4BP was determined by SPR analysis.

An increase in plasmon resonance signal was observed (Fig. 2) when wt-protein S or protein S Heerlen (0-50 nM) was passed over immobilized C4BP (4.5 fmol/mm^2). Binding of both protein S variants appeared to be dose-dependent as the highest response was observed at the highest protein S concentration added. Replacement of protein S solution by buffer resulted in a very slow decline of the response, demonstrating that C4BP-protein S complex formation is reversible and that protein S slowly dissociated from C4BP. To obtain the dissociation rate constants of C4BP-protein S complex, saturating amounts of protein S (500 nM) were added, and dissociation was monitored for a prolonged period of time. Analysis of these data revealed similar dissociation rate constants (k_{off}), association rate constants (k_{on}), stoichiometry (R_{max}) and dissociation constants (K_d) for wt-protein S and protein S Heerlen (Table 1). These data show that wt-protein S and protein S Heerlen are similar with regard to their interaction with C4BP.

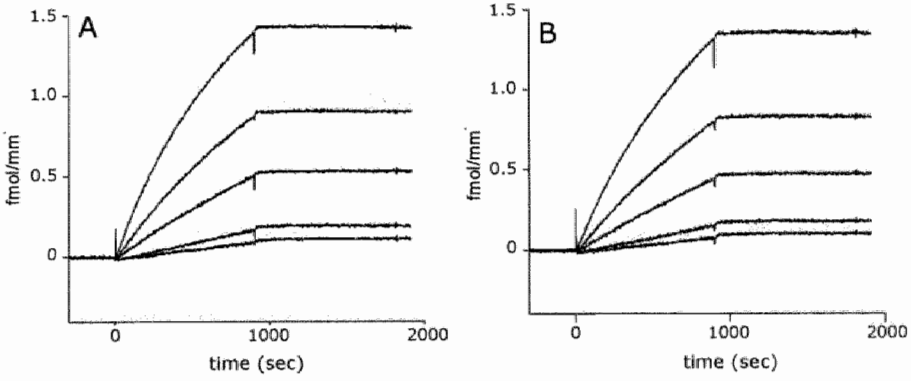

Figure 2: Binding of wt-protein S and protein S Heerlen to immobilized C4BP.

Association phase of wt-protein S (panel A) or protein S Heerlen (panel B) in concentrations of 2, 4, 12.5, 25, 50 nM (bottom to top tracing) to immobilized C4BP monitored in the presence of 3 mM $CaCl_2$ at a flow rate of 5 µl/min. At 900 sec, flow of protein S over the sensor chip was stopped and followed by prolonged flow of buffer (as indicated in the material and methods section).

Table 1: Parameters of the interaction of C4BP with wt-protein S and protein S Heerlen.

	Rmax ($fmol/mm^2$)	k_{off} ($x10^{-6}\ s^{-1}$)	k_{on} ($x10^{4}\ M^{-1}s^{-1}$)	K_D ($x10^{-10}$ M)
wt-protein S	3.20 ± 0.04	5.5 ± 2.0	2.4 ± 0.93	2.7 ± 0.77
Protein S Heerlen	3.29 ± 0.07	5.8 ± 2.0	1.8 ± 0.39	3.3 ± 0.64

Data displayed in this table are mean ± SD of 3 independent measurements.

APC-independent anticoagulant activity of wt-protein S and protein S Heerlen

The APC-independent activities of wt-protein S and protein S Heerlen were compared in a prothrombinase-based assay. wt-protein S and protein S Heerlen inhibited prothrombinase activity to the same extent (Fig. 3). Preparations of protein S were reported to contain a small amount of protein S multimers with a high APC-independent anticoagulant activity [21]. Using native PAGE analysis, equal amounts of multimers (approximately 5%) were found in the purified preparations of wt-protein S and protein S Heerlen (data not shown). To avoid the masking of a potential difference between protein S and protein S Heerlen by the presence of multimers, the latter were removed from both protein S preparations by liposome extraction (see materials and methods). Removal of multimers decreased the APC-independent anticoagulant activity of protein S, but this did not result in a difference between wt-protein S and protein S Heerlen (Fig. 3).

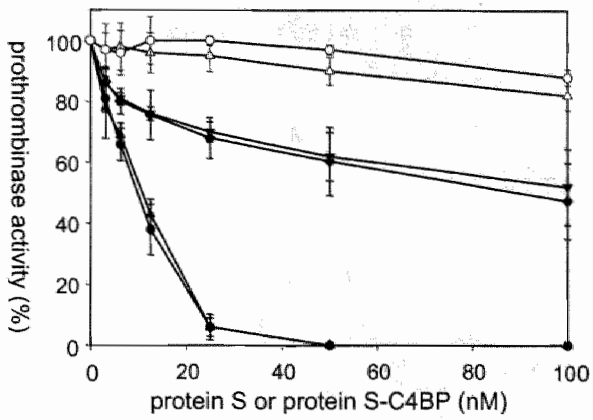

Figure 3: Prothrombinase inhibition by wt-protein S and protein S Heerlen.

Prothrombinase complex (10 pM FXa, 10 pM FVa and 100 nM 20/80 M/M DOPS/DOPC) was incubated in buffer with: wt-protein S (filled triangles), protein S Heerlen (filled circles), multimer-free wt-protein S (open triangles), multimer-free protein S Heerlen (open circles), multimer-free wt-protein S-C4BP complex (filled inverted triangles) or multimer-free protein S Heerlen-C4BP complex (filled diamonds). All protein S variants were added in final concentrations of 3.1, 6.3, 12.5, 25, 50 and 100 nM. Rates of prothrombin activation were determined as described in the materials and methods section. Data points represent mean ± SD of three measurements.

Since individuals carrying the Ser460Pro mutation have very low plasma levels of free protein S Heerlen [24], the protein S-C4BP complex is likely to be the predominantly active form of protein S Heerlen in plasma. Therefore, both "multimer free" preparations of wt-protein S and protein S Heerlen were preincubated for 30 minutes at room temperature at a 1:1.1 molar ratio of C4BP and protein S and APC-independent activities of the complexes were compared. The complexes of wt-protein S and protein S Heerlen with C4BP showed almost identical APC-independent activity. Remarkably, complex formation of protein S with C4BP appeared to increase the level of the inhibition approximately 2-2.5 times (Fig. 3). Addition of C4BP alone to prothrombinase did not result in a significant decrease of prothrombinase activity (data not shown).

Cofactor activity of wt-protein S and protein S Heerlen in APC-catalyzed FVa degradation

The APC-catalyzed inactivation of FVa was followed in time in the presence and absence of plasma protein S, wt-protein S or protein S Heerlen. The three protein S preparations were equally active as APC-cofactors at a concentration of 150 nM (Fig. 4A) accelerating cleavage at Arg306 approximately 3-fold (Table 2). The APC-cofactor activities of plasma protein S, wt-protein S or protein S Heerlen were also comparable when tested at 75 nM and 450 nM protein S and the cofactor activity was proportional with protein S concentrations (data not shown). Inactivation of FVa_{Leiden} by APC in the absence or presence of 150 nM plasma protein S, wt-protein S or protein S Heerlen did not result in different activities of wt-protein S and protein S Heerlen (Fig. 4B), although the acceleration of cleavage at Arg306 was somewhat increased when plasma protein S was used as a cofactor (Table 2). No significant difference between plasma protein S, wt-protein S or protein S Heerlen could be found when the inactivation of FVa_{Leiden} was carried out in the presence of sub-optimal concentrations of protein S (<40 nM, data not shown).

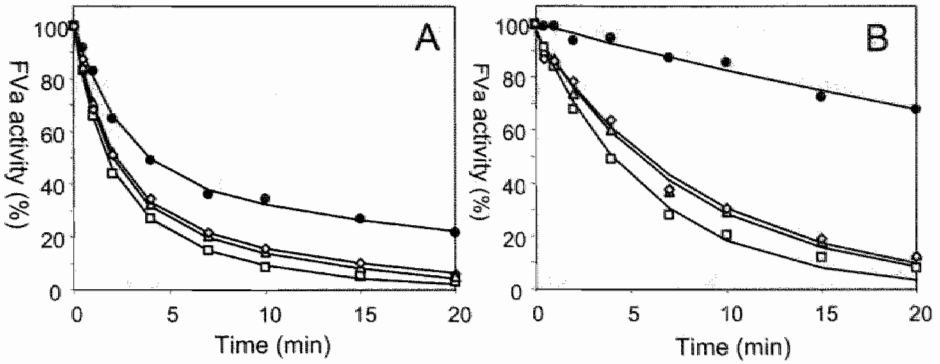

Figure 4: Time courses of normal FVa or FVa_{Leiden} inactivation by APC in the absence or presence of protein S.

The inactivation of normal 0.42 nM FVa (panel A) or 0.42 nM FVa_{Leiden} (panel B) by 0.13 nM APC was followed in time in the absence (filled circles) or presence of 150 nM plasma protein S (open squares), 150 nM wt-protein S (open triangles) or 150 nM protein S Heerlen (open diamonds) in reaction mixtures that contained 40 µM 10/90 DOPS/DOPC vesicles and 3 mM $CaCl_2$. FVa was quantified by measuring its cofactor activity in a prothrombinase-based FVa assay. Solid lines represent the theoretical fits according to the model described in the materials and methods section. Data points represent mean of two measurements.

Table 2: Rate constants of APC-catalyzed FVa inactivation in the absence and presence of protein S.

	FVa normal			FVa Leiden	
	k506 ($x10^7$ $M^{-1}s^{-1}$)	k306 ($x10^7$ $M^{-1}s^{-1}$)	acceleration of k306	k306' ($x10^7$ $M^{-1}s^{-1}$)	acceleration of k306'
No protein S	5.68	0.41		0.16	
Protein S ERL	7.85	1.89	4.6	2.06	13
wt-protein S	6.50	1.31	3.2	1.49	9.1
Protein S Heerlen	6.99	1.22	2.9	1.40	8.5

Data displayed in this table are the averages of at least 2 independent measurements.

APC cofactor activity of protein S in the inactivation of FVIIIa

The APC-cofactor activities of wt-protein S and protein S Heerlen in APC-catalyzed FVIIIa inactivation were tested in a FVIIIa degradation assay both in the absence and presence of FV, which like protein S acts as a cofactor in this reaction [12,13]. Addition of APC to factor VIII caused a reduction of the FIXa cofactor-activity of FVIIIa of 27% over the course of the experiment. In the absence of FV, wt-protein S and protein S Heerlen stimulated factor VIIIa inactivation by APC in a dose-dependent manner, but no difference between both forms of protein S was observed (figure 5). In the presence of FV, wt-protein S and protein S Heerlen again showed identical cofactor activity although the effect of both protein S preparations was enhanced up to four-fold (figure 5). In the absence of APC, protein S did not have any effect on FVIIIa activity (data not shown). After preincubation of plasma protein S, wt-protein S and protein S Heerlen with C4BP (1:2 molar ratio protein S: C4BP), the protein S cofactor activity for FVIIIa-inactivation by APC remained but no synergistic cofactor activity of FV could be observed for each of the protein S-C4BP preparations [14](data not shown).

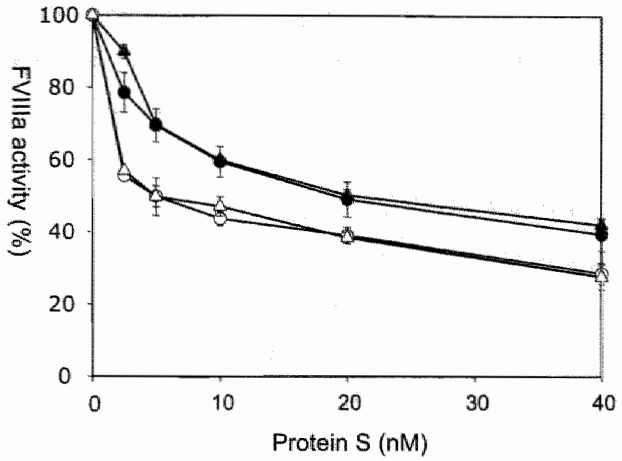

Figure 5: Cofactor activity of wt-protein S and Heerlen in the APC-catalyzed FVIIIa inactivation in the absence or presence of FV.

FVIII was incubated at 37°C with protein S, APC and phospholipids in the absence (filled symbols) or presence (open symbols) of FV. After 5 min a mixture of FIXa, FX, thrombin and calcium chloride was added and 5 min later the amount of FXa formed, which is a measure for the remaining FVIIIa activity in the reaction mixture, was quantified with a FXa-specific chromogenic substrate. Final concentrations of wt-protein S (circles) and Heerlen (triangles) were 0, 2.5, 5, 10, 20 and 40 nM. Further details are given in the materials and methods section. Data points are presented as mean ± SD of 3 experiments and as percentages relative to the FVIIIa activity in the presence of only APC.

Discussion

The Heerlen polymorphism of protein S is characterized by a Ser460Pro substitution and thus lacks glycosylation at Asn458. In the present study recombinant protein S Heerlen (Ser460Pro) was biochemically and functionally compared with recombinant wt-protein S and plasma-derived normal protein S. The HEK293 cell system was well suited to express recombinant proteins that are heavily posttranslationally modified, the gamma-carboxylation was correct and the recombinant protein S molecules appeared to be in the correct 3-dimensional conformation, as shown by their binding to C4BP, their APC-cofactor activity and their APC-independent anticoagulant activity.

Protein S Heerlen showed an increased migration behavior on SDS-PAGE, indicating a reduced mass due to the absence of glycosylation at Asn458. A decrease in mass of approximately 3 kDa for protein S Heerlen was confirmed by MALDI-TOF mass-spectral analysis.

Protein S Heerlen and wt-protein S bound to immobilized C4BP with high affinity and no significant difference between dissociation constants was found for protein S Heerlen (K_d = 0.33 ± 0.06 nM) and wt-protein S (K_d = 0.27 ± 0.08 nM). The observed dissociation constants were similar to that reported for the plasma protein S-C4BP complex (K_d = 0.1 nM) [3]. Using surface plasmon resonance, binding profiles of wt-protein S and protein S Heerlen were observed to be identical and both profiles allowed a correct fit on the basis of a 1:1 stoichiometry for protein S binding to C4BP. Thus, the free protein S deficiency associated with the protein S Heerlen mutation can not be explained by differences in binding stoichiometry to C4BP. Several other hypotheses have been postulated to explain the low levels of free protein S in carriers with the Ser 460 Pro mutation. Being an altered variant of protein S, the protein may be subject to accelerated clearance from circulation [26,32] and protein S Heerlen has been found to be somewhat less heat-stable than its normal counterpart [29]. Secretion differences between wt-protein S and protein S Heerlen were not found [29]. Factors other than protein S Heerlen may also be involved, for example a mutation in the β-chain of C4BP co-segregating with the Ser460Pro mutation leading to decreased free protein S levels, but no abnormalities in the gene coding for the C4BP β-chain were found in a study investigating this gene in 35 carriers of the Ser460Pro mutation [38]. In addition, no linkage with the genes coding for the C4BP α- and β-chain was found in two families carrying the Heerlen allele [25]. One intriguing explanation that has been postulated is possible co-segregation of genes that regulate C4BP β-chain expression and the Ser460Pro mutation [25], but further studies are needed to confirm this.

Recombinant wt-protein S and protein S Heerlen inhibited prothrombinase complex activity equally well in the absence of APC when measured in a purified system. Purified protein S preparations have been found to contain a multimeric fraction, which is most likely an *in vitro* phenomenon. These multimers are high molecular weight, aggregated protein S forms with very high affinity for negatively charged phospholipids and the sole mechanism of prothrombinase inhibition is competition for phospholipid surface [21]. Multimers were also found in wt-protein S and protein S Heerlen in similar amounts. After the protein S multimers present in the purified protein S preparations were removed, both multimer-free wt-protein S and protein S Heerlen were shown to be very weak prothrombinase inhibitors, however, no difference between the two forms of protein S was observed. Interestingly, binding of multimer-free protein S to C4BP increased prothrombinase inhibition approximately 5-fold. It has been reported that the complex of protein S and C4BP retains its APC-independent anticoagulant activity [16,39], but the mechanism of the increased anticoagulant activity of the C4BP-protein S complex that was observed in the present study remains to be elucidated.

Recombinant protein S showed a similar activity compared to plasma-derived protein S as cofactor in the APC-catalyzed inactivation of both normal FV and FV_{Leiden} and no difference between wt-protein S and protein S Heerlen could be observed. Thus, the reported impaired APC-cofactor function of protein S Heerlen in the inactivation of FVa_{Leiden} by APC [29] was not confirmed. No explanation could be found for the observation of Giri et al. [29] that, compared with wt-protein S, protein S Heerlen is over 100-fold less effective as cofactor in the inactivation of FV_{Leiden} by APC. Since the Arg506Gln mutation in FV_{Leiden} does not result in major conformational changes in FVa and does not affect its functional properties such as the cofactor activity in prothrombin activation and the rate constant for cleavage at Arg306 both with and without protein S, it is difficult to visualize that the Ser460Pro mutation in protein S Heerlen would specifically affect the interaction with FV_{Leiden} and not with FV.

wt-protein S and Heerlen displayed equal APC cofactor activities in the inactivation of FVIIIa, either free or in complex with C4BP. The presence of 0.5 nM FV enhanced APC-cofactor activity of both wt-protein S and Heerlen four-fold, demonstrating that protein S Heerlen can function as a normal cofactor for APC in the presence and absence of FV [29]. Under the conditions used in the FVIIIa inactivation assay, no APC-independent effects of protein S were observed.

Protein S-deficiency is a complex disorder and the free protein S-deficiency associated with the Ser460Pro mutation is no exception. For example, the Ser460Pro mutation has not been found in 17 families with quantitative protein S-deficiency [40] and it has been shown not to co-segregate with free protein S-deficiency in three families [25]. In fact, the whole protein S gene (PROS1) was found not to be linked with a protein S-deficient phenotype in one family [25]. This indicated that free protein S-deficiency is, at least in some cases, not inherited in a Mendelian fashion and that other factors contribute to the penetrance of the phenotype. Factors such as age and β-chain C4BP were found to be responsible for the alteration of total protein S-deficiency into free protein S-deficiency [40] and recently the chromosomal region containing the C4BP genes have been found to be in linkage with plasma free protein S levels [41]. Nevertheless, it appears well established now that the Ser460Pro mutation in protein S Heerlen is associated with low levels of free protein S Heerlen and (low) normal levels of protein S-C4BP complex [24-26]. Despite reduced levels of free protein S in carriers of protein S Heerlen, there are thus far no reports that this mutation is associated with an increased risk of venous thrombosis. The present *in vitro* study indicates that the lack of thrombotic complications in carriers of protein S Heerlen cannot be explained by an enhanced anticoagulant activity of protein S Heerlen or the protein S Heerlen-C4BP complex. The possible presence of an unrecognized plasma factor that selectively enhances the anticoagulant activity of protein S Heerlen, either in the absence or presence of APC, remains to be elucidated.

Based on our observations with purified proteins it appears that the mild reduction of the plasma levels of free protein S in heterozygous carriers of protein S Heerlen (~ 60 % of that of normal individuals) in combination with normal levels of total protein S does not sufficiently disturb the overall anticoagulant activity of protein S (APC cofactor and APC independent anticoagulant activity) to give rise to thrombotic complications.

References

1. Comp PC, Nixon RR, Cooper MR, Esmon CT. Familial protein S deficiency is associated with recurrent thrombosis. J Clin Invest. 1984;74:2082-2088
2. Faioni E, Valsecchi C, Palla A, Taioli E, Razzari C, Mannucci P. Free protein S deficiency is a risk factor for venous thrombosis. Thromb Haemost. 1997;78:1343-1346.
3. He X, Shen L, Malmborg A, Smith K, Dahlbäck B, Linse S. Binding site for C4b-binding protein in vitamin K-dependent protein S fully contained in carboxy-terminal laminin-G-type repeats. A study using recombinant factor IX-protein S chimeras and surface plasmon resonance. Biochemistry. 1997;36:3745-3754.
4. Dahlbäck B. Purification of human C4b-binding protein and formation of its complex with vitamin K-dependent protein S. Biochem J. 1983;209:847-856
5. Walker FJ, Chavin SI, Fay PJ. Inactivation of factor VIII by activated protein C and protein S. Arch Biochem Biophys. 1987;252:322-328
6. Koedam JA, Meijers JC, Sixma JJ, Bouma BN. Inactivation of human factor VIII by activated protein C. Cofactor activity of protein S and protective effect of von Willebrand factor. J Clin Invest. 1988;82:1236-1243
7. Walker FJ. Regulation of activated protein C by protein S. The role of phospholipid in factor Va inactivation. J Biol Chem. 1981;256:11128-11131
8. Rosing J, Hoekema L, Nicolaes GA, Thomassen MC, Hemker HC, Varadi K, Schwarz HP, Tans G. Effects of protein S and factor Xa on peptide bond cleavages during inactivation of factor Va and factor VaR506Q by activated protein C. J Biol Chem. 1995;270:27852-27858
9. Norstrom EA, Steen M, Tran S, Dahlback B. Importance of protein S and phospholipid for activated protein C-mediated cleavages in factor Va. J Biol Chem. 2003;278:24904-24911
10. Deguchi H, Fernandez JA, Griffin JH. Neutral glycosphingolipid-dependent inactivation of coagulation factor Va by activated protein C and protein S. J Biol Chem. 2002;277:8861-8865
11. Norstrom E, Thorelli E, Dahlback B. Functional characterization of recombinant FV Hong Kong and FV Cambridge. Blood. 2002;100:524-530

12. Shen L, Dahlbäck B. Factor V and protein S as synergistic cofactors to activated protein C in degradation of factor VIIIa. J Biol Chem. 1994;269:18735-18738
13. Varadi K, Rosing J, Tans G, Pabinger I, Keil B, Schwarz HP. Factor V enhances the cofactor function of protein S in the APC-mediated inactivation of factor VIII: influence of the factor VR506Q mutation. Thromb Haemost. 1996;76:208-214
14. van de Poel RH, Meijers JC, Bouma BN. C4b-binding protein inhibits the factor V-dependent but not the factor V-independent cofactor activity of protein S in the activated protein C-mediated inactivation of factor VIIIa. Thromb Haemost. 2001;85:761-765
15. Heeb MJ, Mesters RM, Tans G, Rosing J, Griffin JH. Binding of protein S to factor Va associated with inhibition of prothrombinase that is independent of activated protein C. J Biol Chem. 1993;268:2872-2877
16. Hackeng TM, van 't Veer C, Meijers JC, Bouma BN. Human protein S inhibits prothrombinase complex activity on endothelial cells and platelets via direct interactions with factors Va and Xa. J Biol Chem. 1994;269:21051-21058
17. Hackeng TM, Hessing M, van 't Veer C, Meijer-Huizinga F, Meijers JC, de Groot PG, van Mourik JA, Bouma BN. Protein S binding to human endothelial cells is required for expression of cofactor activity for activated protein C. J Biol Chem. 1993;268:3993-4000
18. Heeb MJ, Rosing J, Bakker HM, Fernandez JA, Tans G, Griffin JH. Protein S binds to and inhibits factor Xa. Proc Natl Acad Sci U S A. 1994;91:2728-2732
19. Heeb MJ, Kojima Y, Rosing J, Tans G, Griffin JH. C-terminal residues 621-635 of protein S are essential for binding to factor Va. J Biol Chem. 1999;274:36187-36192
20. van Wijnen M, Stam JG, van't Veer C, Meijers JC, Reitsma PH, Bertina RM, Bouma BN. The interaction of protein S with the phospholipid surface is essential for the activated protein C-independent activity of protein S. Thromb Haemost. 1996;76:397-403
21. Sere KM, Janssen MP, Willems GM, Tans G, Rosing J, Hackeng TM. Purified protein S contains multimeric forms with increased APC-independent anticoagulant activity. Biochemistry. 2001;40:8852-8860
22. Dahlbäck B. Inhibition of protein Ca cofactor function of human and bovine protein S by C4b-binding protein. J Biol Chem. 1986;261:12022-12027
23. Bertina R, Ploos van Amstel H, van Wijngaarden A, Coenen J, Leemhuis M, Deutz-Terlouw P, van der Linden I, Reitsma P. Heerlen polymorphism of protein S, an immunologic polymorphism due to dimorphism of residue 460. Blood. 1990;76:538-548.
24. Duchemin J, Gandrille S, Borgel D, Feurgard P, Alhenc-Gelas M, Matheron C, Dreyfus M, Dupuy E, Juhan-Vague I, Aiach M. The Ser 460 to Pro substitution of the protein S alpha (PROS1) gene is a frequent mutation associated with free protein S (type IIa) deficiency. Blood. 1995;86:3436-3443

25. Espinosa-Parrilla Y, Morell M, Souto JC, Borrell M, Heine-Suner D, Tirado I, Volpini V, Estivill X, Sala N. Absence of linkage between type III protein S deficiency and the PROS1 and C4BP genes in families carrying the protein S Heerlen allele. Blood. 1997;89:2799-2806
26. Espinosa-Parrilla Y, Navarro G, Morell M, Abella E, Estivill X, Sala N. Homozygosity for the protein S Heerlen allele is associated with type I PS deficiency in a thrombophilic pedigree with multiple risk factors. Thromb Haemost. 2000;83:102-106.
27. Borgel D, Duchemin J, Alhenc-Gelas M, Matheron C, Aiach M, Gandrille S. Molecular basis for protein S hereditary deficiency: genetic defects observed in 118 patients with type I and type IIa deficiencies. The French Network on Molecular Abnormalities Responsible for Protein C and Protein S Deficiencies. J Lab Clin Med. 1996;128:218-227
28. Morboeuf O, Borgel D, Aiach M, Kaabache T, Gandrille S, Gaussem P. Expression and characterization of recombinant protein S with the Ser 460 Pro mutation. Thromb Res. 2000;100:81-88
29. Giri TK, Yamazaki T, Sala N, Dahlbäck B, de Frutos PG. Deficient APC-cofactor activity of protein S Heerlen in degradation of factor Va Leiden: a possible mechanism of synergism between thrombophilic risk factors. Blood. 2000;96:523-531
30. Lu D, Xie R, Rydzewski A, Long G. The effect of N-linked glycosylation on molecular weight, thrombin cleavage, and functional activity of human protein S. Thromb Haemost. 1997;77:1156-1163.
31. Giri TK, Linse S, Garcia de Frutos P, Yamazaki T, Villoutreix BO, Dahlback B. Structural requirements of anticoagulant protein S for its binding to the complement regulator C4b-binding protein. J Biol Chem. 2002;277:15099-15106
32. Bertina R. XVI congress of the ISTH. Florence, Italy; 1997
33. Bakker HM, Tans G, Janssen-Claessen T, Thomassen MC, Hemker HC, Griffin JH, Rosing J. The effect of phospholipids, calcium ions and protein S on rate constants of human factor Va inactivation by activated human protein C. Eur J Biochem. 1992;208:171-178
34. Sarkar G, Sommer SS. The "megaprimer" method of site-directed mutagenesis. Biotechniques. 1990;8:404-407
35. Nicolaes GA, Tans G, Thomassen MC, Hemker HC, Pabinger I, Varadi K, Schwarz HP, Rosing J. Peptide bond cleavages and loss of functional activity during inactivation of factor Va and factor VaR506Q by activated protein C. J Biol Chem. 1995;270:21158-21166
36. Giri TK, Hillarp A, Hardig Y, Zoller B, Dahlback B. A new direct, fast and quantitative enzyme-linked ligandsorbent assay for measurement of free protein S antigen. Thromb Haemost. 1998;79:767-772
37. Horn IR, van den Berg BMM, van der Meijden PZ, Pannekoek H, van Zonneveld A-J. Molecular Analysis of Ligand Binding to the Second Cluster of Complement-type Repeats of the Low Density Lipoprotein Receptor-related Protein. Evidence for an Allosteric Component in Receptor-Associated Protein-Mediated Inhibition of Ligand Binding. J. Biol. Chem. 1997;272:13608-13613

38. Morboeuf O, Aiach M, Gandrille S. Lack of sequence variations in the C4b-BP beta-chain in patients with type III protein S deficiency bearing the Ser 460 to Pro mutation: description of two new intragenic isomorphisms in the C4b-BP beta-chain gene (C4BPB). Br J Haematol. 1998;101:10-15
39. Koenen RR, Tans G, van Oerle R, Hamulyak K, Rosing J, Hackeng TM. The APC-independent anticoagulant activity of protein S in plasma is decreased by elevated prothrombin levels due to the prothrombin G20210A mutation. Blood. 2003;102:1686-1692
40. Simmonds R, Zöller B, Ireland H, Thompson E, García de Frutos P, Dahlbäck B, Lane D. Genetic and Phenotypic Analysis of a Large (122-Member) Protein S-Deficient Kindred Provides an Explanation for the Familial Coexistence of Type I and Type III Plasma Phenotypes. Blood. 1997;89:4364-4370
41. Almasy L, Soria JM, Souto JC, Coll I, Bacq D, Faure A, Mateo J, Borrell M, Munoz X, Sala N, Stone WH, Lathrop M, Fontcuberta J, Blangero J. A quantitative trait locus influencing free plasma protein S levels on human chromosome 1q: results from the Genetic Analysis of Idiopathic Thrombophilia (GAIT) project. Arterioscler Thromb Vasc Biol. 2003;23:508-511

Chapter 4

Direct anticoagulant activity of protein S-C4b binding protein complex in Heerlen heterozygotes and normals

Heeb MJ, Koenen RR, Fernández JA and Hackeng TM

Journal of Thrombosis and Haemostasis, 2004, 2, 1766 - 1773

Abstract

Background: Plasma protein S normally circulates free (40%) or complexed with C4b-binding protein (PS-C4BP); only free protein S is a cofactor for activated protein C during factor (F) Va inactivation. Protein S-Heerlen lacks a carbohydrate group, leading to low plasma free protein S levels, but normal levels of PS-C4BP. *Objectives:* Because protein S-Heerlen is not associated with thrombosis, we investigated whether PS-C4BP is directly anticoagulant in plasma and whether PS-Heerlen-C4BP has enhanced direct anticoagulant activity. *Methods:* An assay for protein S direct activity was applied to Heerlen-heterozygous plasmas. Free and complexed protein S were repeatedly isolated from normal and Heerlen-heterozygous plasmas and tested for direct anticoagulant activity in prothrombinase assays and in plasma. *Results:* Heerlen-heterozygous plasmas were deficient in free and total protein S antigen but had normal to high protein S direct anticoagulant activity. Purified Heerlen-heterozygous PS-C4BP was 7-fold more potent than normal PS-C4BP in inhibiting full prothrombinase activity, and 22-fold more potent in inhibiting prothrombin activation in the absence of FVa; it also specifically prolonged plasma clotting times 14-fold more than normal PS-C4BP. Heerlen-heterozygous PS-C4BP did not compete for limiting phospholipids any better than normal PS-C4BP. However, ligand blots and surface plasmon resonance studies showed that Heerlen-heterozygous PS-C4BP bound more avidly to FXa than did normal PS-C4BP (apparent Kd=4.3 nM versus 82 nM). *Conclusions:* Plasma-derived PS-C4BP has direct anticoagulant activity in plasma and in purified systems. Enhanced direct activity of PS-Heerlen-C4BP may compensate for low free protein S levels and low cofactor activity in individuals with protein S-Heerlen.

Introduction

Anticoagulant vitamin K-dependent protein S is an important cofactor for activated protein C (APC) in its inactivation of coagulation FVa and FVIIIa[1,2]. Protein S is also anticoagulant independently of APC, by virtue of its direct binding and inhibition of FVa and FXa, its ability to inhibit FVIIIa and its ability to compete with procoagulant components for limiting phospholipid surfaces [3-8]. 60% of protein S in blood is normally complexed to complement C4b-binding protein (PS-C4BP) and inactive as an APC cofactor during FVa inactivation[9]. However, both free protein S and PS-C4BP can directly inhibit prothrombinase activity [5]. Heterozygous protein S deficiency is associated with increased risk of venous thrombosis and stroke; homozygous deficiency may lead to severe neonatal thrombosis [10-12].

Protein S-Heerlen lacks a carbohydrate attachment group at N458 due to the mutation S460P that disrupts a glycosylation consensus sequence [13,14]. Heterozygotes with the S460P mutation had low free protein S (38-66% of normal) but protein S cofactor activity for APC was proportional to levels of free protein S, suggesting that the variant molecule was not deficient in cofactor activity [13] (RM Bertina, subcommittee meeting, ISTH 1997). Free protein S-Heerlen might be cleared more rapidly than normal protein S, since the low free fraction of protein S in Heerlen heterozygotes consisted mostly of normal protein S. Heterozygotes had normal levels of complexed protein S that were ~50% protein S-Heerlen [13] (Bertina, ISTH 1997).

Among Dutch, 1 of 200 individuals have protein S-Heerlen, with a small but not statistically significant increased proportion in individuals with venous thrombosis [14]. Protein S-Heerlen was found in 16 of 85 symptomatic French patients with protein S deficiency, but in only 1 of 113 healthy subjects [15]. In another study, almost half of symptomatic protein S-deficient patients with protein S-Heerlen had an identifiable thrombotic risk factor [16], while an asymptomatic 20 year-old woman homozygous for protein S-Heerlen had 34-40% free protein S and 50% total protein S [17].

Free protein S levels in Heerlen-heterozygous plasmas are similar to those of thrombophilic individuals with heterozygous deficiency of protein S. Since protein S-Heerlen heterozygotes may not experience significantly increased risk of thrombosis, we hypothesized that PS-C4BP is anticoagulant and/or that protein S-Heerlen has enhanced APC-independent anticoagulant activity. To test this hypothesis, we applied an assay for direct anticoagulant activity of protein S in plasma [18], and we repeatedly isolated and tested free and complexed protein S from two Heerlen-heterozygous and two normal plasmas.

Materials and Methods

Proteins.

FVa, C4BP and prothrombin were prepared as described [19,20]. FXa and thrombin were purchased from Enzyme Research Laboratories, South Bend IN. Serum amyloid P component was from Sigma, St. Louis, MO. Antibodies against protein S were prepared as described [3,10]. Protein S and PS-C4BP were purified [4] using barium adsorption and elution of citrated plasma, DEAE-Sephacel and Blue-Sepharose chromatography (Amersham-Pharmacia, Piscataway NJ).

Platelets.

Platelets were prepared from blood containing 7 mM EDTA/7 mM trisodium citrate, using differential centrifugation [21]. Platelets were activated (1 U/ml thrombin, 10 min) or lysed (1.25% SDS).

ELISAs.

Protein S antigen was assayed [22] using immunoaffinity-purified goat anti-protein S as coating antibody (2 μg/ml) and biotinylated-immunoaffinity-purified goat anti-protein S (1.5 μg/ml) as detecting antibody. Dilutions of pooled normal plasma (George King, Overland Park, KS) served as standards. Free protein S was assayed using monoclonal capture antibody 15C4 and biotinylated detecting antibody 34G2 against free protein S [23] (Stago, Asnières, France), and streptavidin-peroxidase. Free protein S was also assayed with Asserachrome kits (Stago).

Protein S activity assays.

Protein S cofactor activity for APC was determined using Staclot S kits (Stago). Protein S direct anticoagulant activity was determined in prothrombinase assays with and without 20 pM FVa, using 1 nM FXa, 40 μM phospholipid vesicles (phosphatidyl serine/phosphatidyl choline 20/80), and 0.3 μM prothrombin [3]. In some experiments, limiting (0.1-5 μM) or varying phospholipids were used. Protein S was preincubated 10 min with FXa/FVa/phospholipids prior to prothrombin addition. Aliquots were taken over time and prothrombinase was quenched with EDTA; the rate of thrombin generation was measured with amidolytic substrate CBS 34.47 (American Bioproducts, Parsippany, NY).

A FXa-based assay for the direct anticoagulant activity of protein S in plasma [18] was modified by use of monoclonal antibody S4 [24] that neutralizes Heerlen and normal protein S with equal efficiency. Plasma was incubated 3 min in a microtiter plate without/with S4 (1.25 mg/ml) and with neutralizing antibody to APC, followed by addition of FXa/phospholipid vesicles/$CaCl_2$. Clot time was taken as time to half maximal absorbance at 340 nm; ratio of clot times without/with S4 was calculated and compared to ratios for various plasmas.

An APTT assay was used to demonstrate prolongation of clot times by protein S or PS-C4BP. Protein S-depleted plasma and protein S were incubated 2 min with dilute (1:3) APTT reagent; $CaCl_2$ was added and clot time was measured.

Surface plasmon resonance binding measurements.

One channel of a CM-5 chip (Biacore, Piscataway, NJ) was coupled with FXa [25] and one channel was treated the same way without FXa. Using a Biacore 3000, varying concentrations of PS-C4BP were injected at 5 μl/min in 0.02 M HBS, 0.3 M NaCl, 5 mM $CaCl_2$, 0.005% P-40, each followed by buffer wash and regeneration of the chip (3 M NaCl). Sensorgrams representing association/dissociation of PS-C4BP were collected, overlaid and analyzed (Biaevaluation software).

Results

Plasma and platelets from heterozygotes with protein S-Heerlen.

Fortuitously, a volunteer's platelets contained protein S-Heerlen (Fig. 1A). Normal unstimulated platelets (U) contained only one band of single chain protein S under reducing conditions, while unstimulated platelets from the volunteer contained two forms of protein S. Both the normal and the variant protein S in stimulated platelet releasate (S) from the protein S-Heerlen heterozygote were mostly cleaved in the thrombin-sensitive region, and migrated as two bands of increased mobility compared to cleaved and uncleaved protein S from stimulated normal releasate. Genetic analysis confirmed the S460P mutation.

Plasma from the volunteer and her two children (ages 19 to 21 years) were analyzed by immunoblotting (Fig. 1B). Mother and daughter appeared heterozygous for protein S-Heerlen, while the son had normal protein S. The mother's and daughter's plasmas appeared to have less Heerlen-protein S than normal protein S, consistent with reports of low-normal total protein S and low free protein S in Heerlen heterozygotes [17]. None of 5 antibodies tested recognized protein S-Heerlen more efficiently than the goat anti-protein S employed in Figure 1 and Table 1.

The two Heerlen heterozygotes had low-normal or low total protein S and low free protein S levels, and low APC cofactor activity (Table 1). The son had normal protein S and high-normal levels in each category. Interestingly, the daughter had lower protein S antigen and cofactor activity at times when she was taking oral contraceptives (denoted by * in Table 1), which are reported to result in lower protein S levels [26]. The Heerlen-heterozygous plasmas, but not the normal plasma, had 1.3-fold higher APC cofactor activity than expected from free protein S levels.

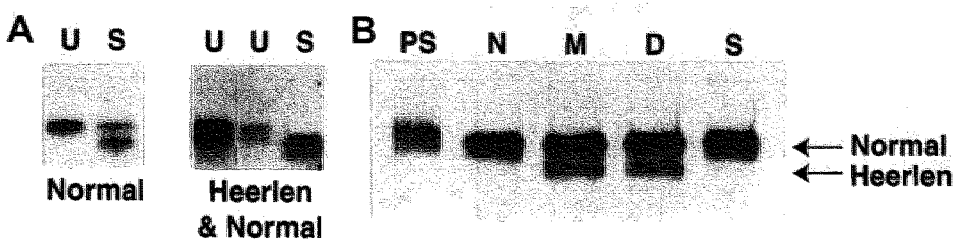

Figure 1. Immunoblots for protein S in platelets and plasmas from a family with Heerlen-heterozygous members. (A) Reduced platelet samples from a normal individual and from a Heerlen heterozygote were immunoblotted with goat anti-protein S. U denotes unstimulated platelets lysed in SDS (lanes 1 and 3) and supernatant from unstimulated platelets (lane 4). S denotes soluble releasate from thrombin-stimulated platelets. Reduced protein S from unstimulated platelets is in single chain form, but the majority of protein S from thrombin-stimulated platelets is cleaved in the thrombin-sensitive loop, resulting in faster mobility on reduced gels. (B) Non-reduced plasma samples. Mother, M, and daughter, D, were confirmed by genetic analysis to be Heerlen heterozygotes; the son, S, had only normal protein S. PS, purified normal protein S; N, pooled normal plasma.

Table 1. Direct anticoagulant activity of plasma protein S compared to other parameters.

Plasma Type	Ratio Clot Time ± mAb.	% Tot. PS Antigen (3)	% Free PS Antigen (3)	% APC Cofactor(3)
Normal (Son)	1.73 (2)	126	121	118
Pooled normal	1.65 (9)	100	100	100
Heerlen-heterozygous (M)	1.92, 1.61 (5)	72, 72	52, 46	67, 54
Heerlen-heterozygous (D)	1.58*, 1.68* (6)	56*, 75	39*, 49.5	50*, 67
Heterozygous PS-deficient 1	1.30 (5)	68	27	30
Heterozygous PS-deficient 2	1.25**	61**		
Heterozygous PS-deficient 3	1.24**	53**		
Heterozygous PS-deficient 4	1.24 (2)	45	16**	17**
Double heteroz. PS-deficient	1.12 (5)	11	≤2	<7
PS-depleted	1.09 (7)	<1**		

Assays are described in Methods. Values are means of the number of assays shown in parentheses. For Heerlen heterozygotes, parameters were measured several times in each of 2 years. *Oral contraceptive use at sampling time. **Only 1 measurement.

Direct anticoagulant activity of protein S in Heerlen plasma.

We developed an assay for protein S direct anticoagulant activity in plasma that showed this activity to be low in plasmas with prothrombin mutation G20210A [18], possibly because high levels of prothrombin competed with protein S for FVa binding [3]. We applied this assay to Heerlen-heterozygous plasmas and to other plasmas (Table 1, column 2). Ratios of clot times without/with antibody S4 for the Heerlen plasmas were higher than expected from their low free and total protein S antigen levels, while ratios for plasmas of other protein S-deficient individuals were proportional to free and total antigen levels, and in the expected order compared to normal plasma. Heerlen plasmas had 116-119% levels of prothrombin in a prothrombin time assay, futher suggesting enhanced direct activity of protein S-Heerlen, since increased prothrombin levels normally lead to low protein S direct activity measurements [18]. Thus, native protein S-Heerlen in plasma appears more active than normal protein S, and PS-C4BP is suggested to have direct anticoagulant activity.

Purification of free and complexed protein S from Heerlen heterozygotes and normals.

Protein S and PS-C4BP from one unit of plasma from each of the two Heerlen heterozygotes were isolated 4 times over a 3-year period. Normal protein S was isolated in an identical manner. On SDS-PAGE (Fig. 2), protein S-Heerlen was detected as a band just below the band for normal protein S, resulting in the appearance of a broader protein S band. The greater-mobility band was identified as protein S through sequencing.

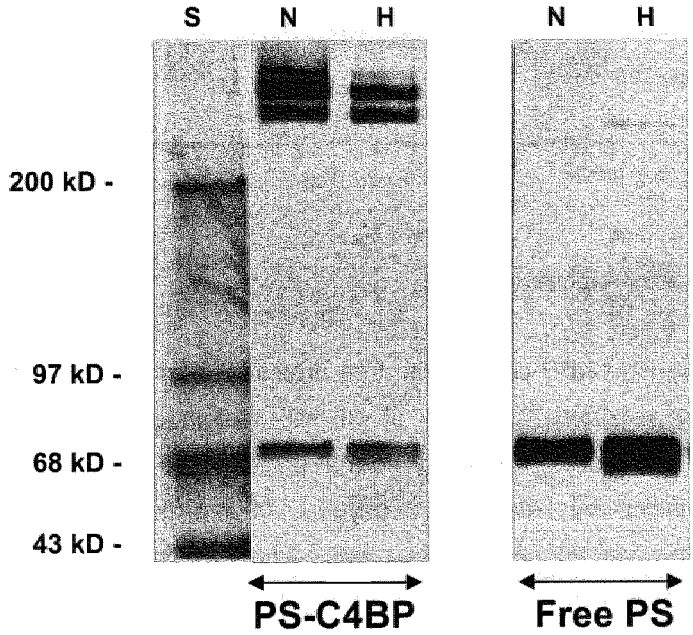

Figure 2. SDS-PAGE of purified free and complexed protein S from Heerlen heterozygotes and normals. Nonreduced 7% gel. (S), molecular weight standards; (N), normal protein S; (H), Heerlen-heterozygous protein S.

Prothrombinase inhibitory activity of purified protein S.

Purified Heerlen-heterozygous PS-C4BP was more potent than normal PS-C4BP in its ability to inhibit the full prothrombinase complex (Fig. 3A). Its ability to inhibit thrombin generation was more potent in the absence of FVa (Fig. 3B), while normal PS-C4BP was less potent when FVa was omitted.

These results were consistent in 4 different heterozygous and 3 different normal preparations (Table 2). Concentrations of protein S needed for 50% inhibition of full prothrombinase were 7-fold less for the heterozygous PS-Heerlen-C4BP mixtures than for normal PS-C4BP, and 22 times less for inhibition of thrombin generation in the absence of FVa (Table 2). Kinetic analysis showed that both types of PS-C4BP were noncompetitive inhibitors of FXa in the absence of FVa (data not shown).

Table 2. Direct anticoagulant activity of protein S preparations.

Protein S Preparations	**[PS] for 50% Inhibition of Prothrombinase (nM)**	
	With FVa	Without FVa
PS-C4BP (H + N), 4 preps.	9.6 ± 5.2 (S.D.)	4.9 ± 4.1 (S.D.)
PS-C4BP (N), 3 preps.	68 ± 32	107 ± 34

H+N, Heerlen-heterozygous; N, normal.

Unlike PS-C4BP complexes, free protein S preparations varied greatly in activity, as reported [3]. Activity of all free protein S preparations ranged between the depicted inhibition by normal protein S (Fig. 3A, open squares) and the inhibition by Heerlen-heterozygous free protein S (Fig. 3A, open triangles). However, Heerlen-heterozygous free protein S probably contains more normal than Heerlen protein S (Bertina, ISTH 1997).

Contaminants of protein S preparations that are sometimes observed are prothrombin and serum amyloid P component [27]. To determine whether these might be responsible for enhanced activity of PS-Heerlen-C4BP heterozygous mixtures, serum amyloid P component or prothrombin were added at equimolar concentrations to purified complexed and free normal protein S and heterozygous protein S-Heerlen. Neither component affected protein S inhibition of prothrombinase (data not shown) and it seems unlikely that a confounding contaminant was present in 4 Heerlen-heterozygous preparations, but not in 3 normal preparations.

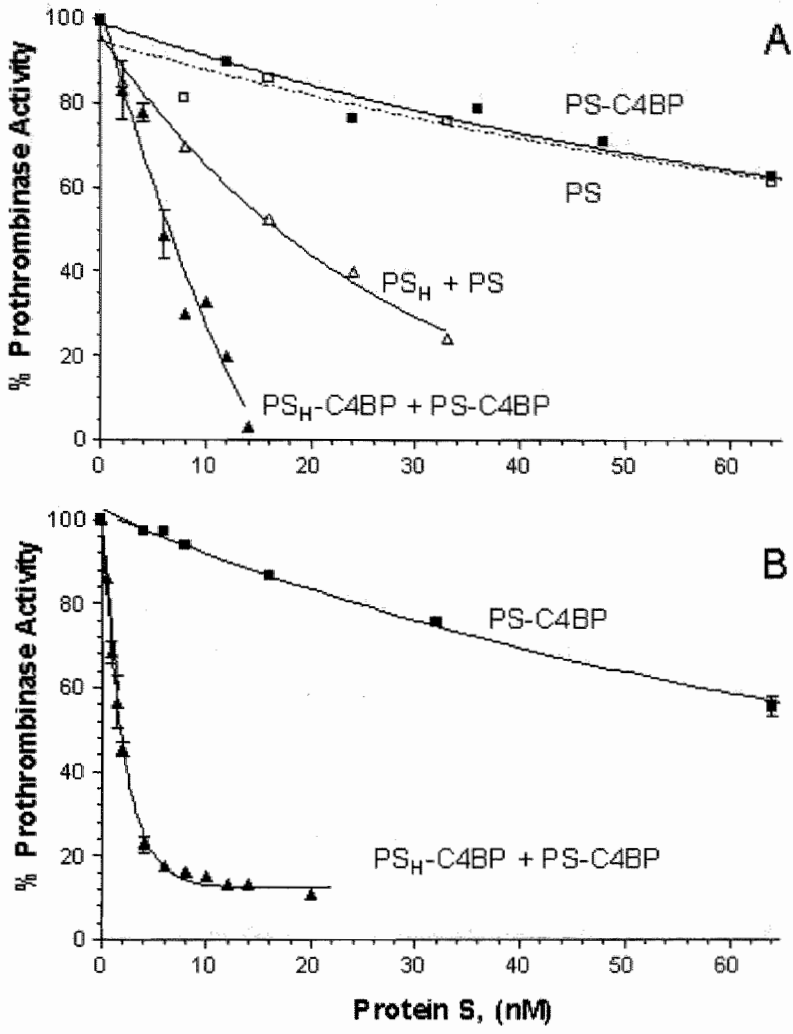

Figure 3. Inhibition of prothrombinase activity by purified normal and Heerlen-heterozygous PS-C4BP. (A) In the presence of FVa; (B) In the absence of FVa. Prothrombinase assays were performed as described. Free normal protein S (open squares), free Heerlen-heterozygous protein S (open triangles), normal PS-C4BP (filled squares) and Heerlen-heterozygous PS-C4BP (filled triangles) were tested for their ability to inhibit prothrombinase. Data are representative of over 10 experiments.

We investigated whether complexation with C4BP could enhance the ability of free protein S to inhibit prothrombinase. Normal or Heerlen-heterozygous protein S was preincubated with a 1.3-fold molar excess of C4BP. APC cofactor activity after incubation was <20% of its original activity, suggesting that most of the protein S was complexed. Complexation of each protein S preparation with C4BP modestly enhanced its prothrombinase inhibitory activity (1.4 to 4-fold). That enhancement alone did not fully account for the 7 to 22-fold greater than normal anticoagulant activity of Heerlen-heterozygous PS-C4BP.

Reconstitution of protein S-depleted plasma with PS-C4BP.

Enhanced direct anticoagulant activity of Heerlen-heterozygous plasmas (Table 1) could be due to either free or complexed protein S-Heerlen, but PS-C4BP is not considered to be anticoagulant in plasma. To test this, purified free and complexed protein S were added to protein S-depleted plasma and to normal plasma. Both normal and Heerlen-heterozygous PS-C4BP prolonged APTT clotting times, but the Heerlen-heterozygous mixture was 14-fold more effective (Fig. 4). Heerlen-heterozygous free protein S prolonged clotting time less than did Heerlen-heterozygous PS-C4BP, but about 4-fold more than free normal protein S. Prolongations were specific for protein S because they were blocked by antibody S4.

Relative activities of these protein S species in the APTT assays were similar to their relative activities in prothrombinase assays, suggesting that no component in normal (not shown) or protein S-depleted plasma could enhance the activity of the less active protein S species.

Removal of N-linked carbohydrate from protein S.

We investigated the effect of removal of all N-linked carbohydrates from normal protein S. Protein S had maximum increase in mobility on SDS-PAGE after 16 h of glycosidase treatment, suggesting that all N-linked carbohydrate was removed. Treated protein S did not have increased APC cofactor activity or direct activity compared to protein S treated the same way without glycosidases (data not shown).

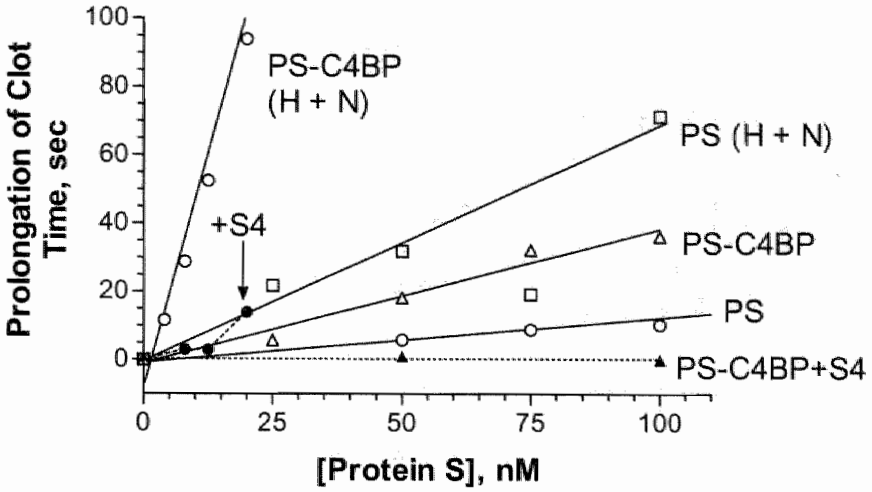

Figure 4. Prolongation of clotting times by PS-C4BP.
Purified normal or Heerlen-heterozygous PS-C4BP was preincubated 30 min in PS-depleted plasma in the absence (open symbols) or presence (closed symbols and dashed lines) of 0.5 mg/ml neutralizing anti-protein S antibody S4. APTT was measured as described in Methods. Data are representative of three experiments.

Effect of varying phospholipid on inhibition of prothrombinase by protein S.

Since one mechanism for protein S anticoagulant activity is competition for limiting phospholipids [7], we tested the ability of protein S or PS-C4BP to inhibit prothrombinase at variable phospholipid concentrations. At lower phospholipid concentrations (<10 μM), inhibitory effects of protein S and PS-C4BP were greater, likely due to protein S competition with prothrombinase components for phospholipid surfaces (Fig. 5). Heerlen-heterozygous PS-C4BP did not compete for phospholipids better than normal PS-C4BP. At the 40 μM phospholipids used in our experiments, inhibition of prothrombinase was independent of phospholipid concentration, suggesting that primary protein S inhibitory mechanisms in experiments shown in Fig. 3 involved protein S interactions with FXa and/or FVa.

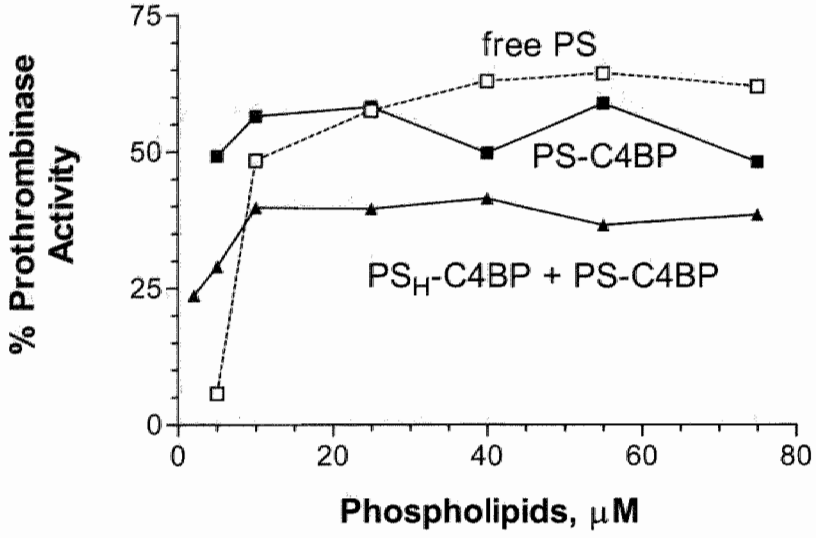

Figure 5. Effect of phospholipid concentration on the ability of protein S to inhibit prothrombinase. Phospholipid vesicles at various concentrations were preincubated for 15 min with FXa/FVa/Ca^{2+} with/without normal protein S (open squares), normal PS-C4BP (filled squares), or Heerlen-heterozygous PS-C4BP (filled triangles). Prothrombin was added and the rate of thrombin generation was measured. 100% activity (0% inhibition) was defined as prothrombinase activity in the absence of protein S at each phospholipid concentration. Data are representative of 4 experiments.

Binding of PS-C4BP to FXa.

The prothrombinase inhibitory activity of Heerlen-heterozygous PS-C4BP was more efficient in the absence of FVa than in the presence of FVa (Fig. 3B). Since protein S binds specifically to FXa on ligand blots [4], we compared the ability of normal and Heerlen-heterozygous free and complexed protein S to bind to varying quantities of FXa on ligand blots. Binding of 3 μg/ml (40 nM) Heerlen-heterozygous PS-C4BP to as little as 6.2 ng of FXa was easily detected, whereas binding of normal PS-C4BP to 50 ng of FXa could barely be detected (Fig. 6A). Heerlen-heterozygous free protein S also bound more avidly to FXa than did normal protein S.

Surface plasmon resonance studies confirmed higher FXa binding affinity for Heerlen-heterozygous PS-C4BP than for normal PS-C4BP (Fig. 6B). Global fits of sensorgrams for association and dissociation data were performed (Table 3). True K_d could not be calculated for the Heerlen-hetrozygous PS-C4BP mixture, but the "apparent" K_d for each type of PS-C4BP was consistent with its IC50 for inhibition of prothrombinase in the absence of FVa (Table 2). Thus, the 19-fold greater affinity for FXa of Heerlen-heterozygous PS-C4BP provides a rationale for its potent direct anticoagulant activity in plasma and purified systems.

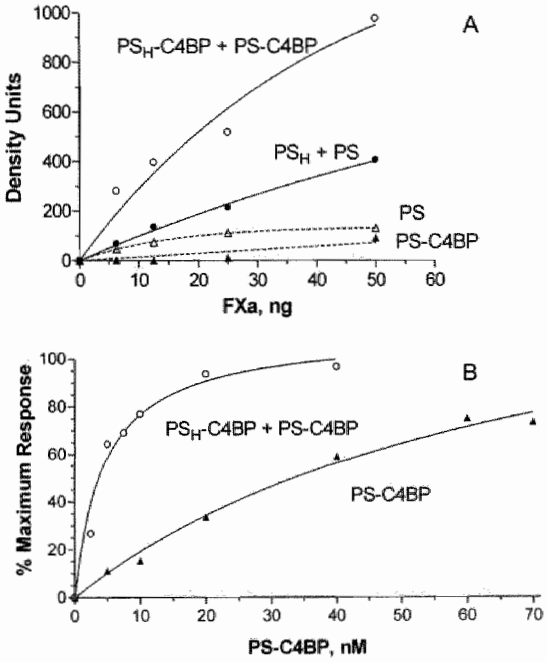

Figure 6. Interaction of purified protein S with factor Xa.
(A) Ligand blotting of FXa with protein S. Varying amounts of FXa were electrophoresed and transferred to a membrane. The membrane was blocked and incubated with the protein S species indicated. Bound protein S was detected with monoclonal S8. Bands were scanned by densitometry and the intensities expressed in arbitrary units. (B) Surface plasmon resonance measurements of PS-C4BP binding to FXa. Normal or Heerlen-heterozygous PS-C4BP at various concentrations was injected over 2 channels of a CM5 chip.

Peak binding response for the blank channel was subtracted from peak response for the channel with coupled FXa. A fit of these data yielded half maximal responses at 72 nM normal PS-C4BP and 4.5 nM Heerlen-heterozygous PS-C4BP.

Table 3. Kinetic parameters for binding of PS-C4BP to FXa by surface plasmon resonance.

Protein S Preparation	k off (s^{-1})	k on ($M^{-1}s^{-1}$)	App K_d = k off/ k on
PS-C4BP (H + N)	3.9×10^{-3}	9.0×10^{5}	4.3×10^{-9} M
PS-C4BP (N)	9.9×10^{-3}	1.2×10^{5}	82×10^{-9} M

A global fit of sensorgrams from experiments in Fig. 6B was performed using Biaevaluation software.

Discussion

These studies emphasize the possible physiologic anticoagulant role of PS-C4BP, since PS-C4BP directly inhibited prothrombinase, confirming our previous report [5]. We also report that addition of purified PS-C4BP to plasma prolongs clotting times. Purified Heerlen-heterozygous PS-C4BP had consistently greater direct anticoagulant activity than normal PS-C4BP. Significantly, Heerlen-heterozygous *plasmas* have normal protein S direct activity despite low protein S antigen, whereas other protein S-deficient plasmas with comparable antigen levels had low direct protein S activity. PS-Heerlen-C4BP may have sufficient APC-independent anticoagulant activity to be detected in cofactor assays, since cofactor activities of Heerlen-heterozygous plasmas were 1.3-fold higher than expected from their free protein S levels. Alternatively, free protein S-Heerlen may have somewhat higher than normal cofactor activity, as reported for r-protein S Heerlen [28].

Several potential mechanisms for the increased direct anticoagulant activity of Heerlen-heterozygous PS-Heerlen were investigated. We could not selectively remove the carbohydrate group at N458 from normal protein S, but removal of all N-linked carbohydrate did not result in increased direct anticoagulant activity or APC cofactor activity. Heerlen-heterozygous PS-C4BP did not compete for limited phospholipid better than normal PS-C4BP.

Furthermore, protein S competition for phospholipid was observed only below 10 μM phospholipids, while 40 μM was used in other experiments presented here.

Complexation of purified free normal or Heerlen-heterozygous protein S with C4BP only modestly increased their direct anticoagulant activity (1.4 to 4 fold). Thus, we hypothesize that the direct activity of plasma-derived protein S is protected during purification steps when it is complexed to C4BP. Accordingly, we find that PS-C4BP has consistent direct activity among preparations, while free protein S activity varies greatly.

Enhanced direct anticoagulant activity of Heerlen-heterozygous PS-C4BP purified from plasma was particularly apparent in the absence of FVa, and might be explained by a greater affinity for FXa due to removal of steric hindrance by the carbohydrate chain that is normally attached at N458. Indeed, Heerlen-heterozygous PS-C4BP had greater than normal affinity for FXa. An example of this type of effect was reported for FVa_1/FVa_2 forms, where the form lacking one carbohydrate group in the light chain binds with greater affinity to phospholipids and is more susceptible to APC [29-31]. Yet, removal of all carbohydrates from protein S did not enhance its activities.

We are left with the most likely explanation that the 19-fold higher affinity for Heerlen-heterozygous PS-C4BP for FXa and the modestly enhanced activity of protein S when complexed with C4BP account for the 7 to 22-fold greater than normal ability of Heerlen-heterozygous PS-C4BP to inhibit prothrombinase activity.

We also prepared recombinant wild-type and Heerlen protein S [32]. Neither r-protein S species had as much direct anticoagulant activity as the corresponding plasma-derived proteins and they had similar activities after complexation with C4BP. Recombinant proteins do not always have the same properties as proteins expressed in vivo and an active state of protein S-Heerlen may exist in plasma. This could be due to: a) an active conformation, perhaps induced by the insertion of Pro460, that is protected by C4BP during purification; b) different glycosylation or other posttranslational modifications than those occurring in r-protein S;

c) specific binding of an undetected trace or small molecule that enhances its activity. Plasma did not enhance/restore the activity of normal or Heerlen-heterozygous free protein S, but we cannot exclude that denaturation of protein S-Heerlen during purification rendered it incapable of binding a plasma enhancing component. The nature of these differences may provide clues to physiological mechanisms and requires future investigations. Since Heerlen-heterozygous plasmas exhibit higher direct anticoagulant activity than expected from their protein S antigen levels, an active state may exist in plasma.

r-Protein S-Heerlen and wild-type protein S had similar affinities for C4BP [28,33,34], although the missing carbohydrate chain in protein S-Heerlen is near a binding site for C4BP [35]. One study predicted a 2:1 stoichiometry of r-PS-Heerlen:C4BP [15]. However, we calculated a 1:1 stoichiometry for plasma-derived normal or Heerlen PS:C4BP, consistent with another study [19].

Protein S-Heerlen exists in 0.5-1 % of populations studied, and is therefore of interest as a potential risk factor for venous thrombosis or stroke, due to the type I or type III deficiencies observed with this variant [17]. The anecdotal observation of even lower protein S levels in the daughter in our study when she was taking oral contraceptives suggests that the decrease in protein S observed in women taking oral contraceptives [26] is exacerbated in Heerlen-heterozygous women. However, the lack of clear evidence of increased thrombotic risk in individuals with this variant [14] led us to investigate the direct anticoagulant activity of free and complexed protein S-Heerlen. Present findings are particularly interesting in light of the significantly reduced free protein S levels in plasma from individuals with protein S-Heerlen, since only free protein S is known to possess APC-cofactor activity. This suggests that direct anticoagulant activity of PS-C4BP may be physiologically relevant and we speculate that the increased direct anticoagulant activity of PS-Heerlen-C4BP might compensate for the low levels of free protein S and low APC-cofactor activity of protein S in individuals carrying protein S-Heerlen.

References

1. Walker FJ. Regulation of activated protein C by a new protein. A possible function for bovine protein S. J Biol Chem. 1980;255:5521-5524
2. Koedam JA, Meijers JC, Sixma JJ, Bouma BN. Inactivation of human factor VIII by activated protein C. Cofactor activity of protein S and protective effect of von Willebrand factor. J Clin Invest. 1988;82:1236-1243
3. Heeb MJ, Mesters RM, Tans G, Rosing J, Griffin JH. Binding of protein S to factor Va associated with inhibition of prothrombinase that is independent of activated protein C. J Biol Chem. 1993;268:2872-2877
4. Heeb MJ, Rosing J, Bakker HM, Fernandez JA, Tans G, Griffin JH. Protein S binds to and inhibits factor Xa. Proc Natl Acad Sci U S A. 1994;91:2728-2732
5. Hackeng TM, van 't Veer C, Meijers JC, Bouma BN. Human protein S inhibits prothrombinase complex activity on endothelial cells and platelets via direct interactions with factors Va and Xa. J Biol Chem. 1994;269:21051-21058
6. Koppelman SJ, Hackeng TM, Sixma JJ, Bouma BN. Inhibition of the intrinsic factor X activating complex by protein S: evidence for a specific binding of protein S to factor VIII. Blood. 1995;86:1062-1071
7. van Wijnen M, Stam JG, van't Veer C, Meijers JC, Reitsma PH, Bertina RM, Bouma BN. The interaction of protein S with the phospholipid surface is essential for the activated protein C-independent activity of protein S. Thromb Haemost. 1996;76:397-403
8. van 't Veer C, Butenas S, Golden N, Mann K. Regulation of prothrombinase activity by protein S. Thromb Haemost. 1999;82:80-87.
9. Comp PC, Doray D, Patton D, Esmon CT. An abnormal plasma distribution of protein S occurs in functional protein S deficiency. Blood. 1986;67:504-508
10. Schwarz H, Fischer M, Hopmeier P, Batard M, Griffin J. Plasma protein S deficiency in familial thrombotic disease. Blood. 1984;64:1297-1300.
11. Mahasandana C, Suvatte V, Marlar RA, Manco-Johnson MJ, Jacobson LJ, Hathaway WE. Neonatal purpura fulminans associated with homozygous protein S deficiency. Lancet. 1990;335:61-62
12. Thommen D, Buhrfeind E, Felix R, Sulzer I, Furlan M, Lammle B. [Hemostasis parameters in 55 patients with venous and/or arterial thromboembolisms]. Schweiz Med Wochenschr. 1989;119:493-499
13. Schwarz HP, Heeb MJ, Lottenberg R, Roberts H, Griffin JH. Familial protein S deficiency with a variant protein S molecule in plasma and platelets. Blood. 1989;74:213-221

14. Bertina R, Ploos van Amstel H, van Wijngaarden A, Coenen J, Leemhuis M, Deutz-Terlouw P, van der Linden I, Reitsma P. Heerlen polymorphism of protein S, an immunologic polymorphism due to dimorphism of residue 460. Blood. 1990;76:538-548.
15. Duchemin J, Gandrille S, Borgel D, Feurgard P, Alhenc-Gelas M, Matheron C, Dreyfus M, Dupuy E, Juhan-Vague I, Aiach M. The Ser 460 to Pro substitution of the protein S alpha (PROS1) gene is a frequent mutation associated with free protein S (type IIa) deficiency. Blood. 1995;86:3436-3443
16. Borgel D, Duchemin J, Alhenc-Gelas M, Matheron C, Aiach M, Gandrille S. Molecular basis for protein S hereditary deficiency: genetic defects observed in 118 patients with type I and type IIa deficiencies. The French Network on Molecular Abnormalities Responsible for Protein C and Protein S Deficiencies. J Lab Clin Med. 1996;128:218-227
17. Espinosa-Parrilla Y, Navarro G, Morell M, Abella E, Estivill X, Sala N. Homozygosity for the protein S Heerlen allele is associated with type I PS deficiency in a thrombophilic pedigree with multiple risk factors. Thromb Haemost. 2000;83:102-106.
18. Koenen RR, Tans G, van Oerle R, Hamulyak K, Rosing J, Hackeng TM. The APC-independent anticoagulant activity of protein S in plasma is decreased by elevated prothrombin levels due to the prothrombin G20210A mutation. Blood. 2003;102:1686-1692
19. Griffin J, Gruber A, Fernandez J. Reevaluation of total, free, and bound protein S and C4b-binding protein levels in plasma anticoagulated with citrate or hirudin. Blood. 1992;79:3203-3211
20. Di Scipio RG, Hermodson MA, Yates SG, Davie EW. A comparison of human prothrombin, factor IX (Christmas factor), factor X (Stuart factor), and protein S. Biochemistry. 1977;16:698-706
21. Tans G, Rosing J, Thomassen MC, Heeb MJ, Zwaal RF, Griffin JH. Comparison of anticoagulant and procoagulant activities of stimulated platelets and platelet-derived microparticles. Blood. 1991;77:2641-2648
22. Heeb MJ, Kojima Y, Rosing J, Tans G, Griffin JH. C-terminal residues 621-635 of protein S are essential for binding to factor Va. J Biol Chem. 1999;274:36187-36192
23. Amiral J, Grosley B, Boyer-Neumann C, Marfaing-Koka A, Peynaud-Debayle E, Wolf M, Meyer D. New direct assay of free protein S antigen using two distinct monoclonal antibodies specific for the free form. Blood Coagul Fibrinolysis. 1994;5:179-186
24. Heeb MJ, Mesters RM, Griffin JH. Monoclonal antibody S4 directed against the N-terminal region of protein S blocks protein S inhibition of prothrombinase in the absence of phospholipid. Thromb Haemost. 1999;(suppl):565
25. Mounier CM, Hackeng TM, Schaeffer F, Faure G, Bon C, Griffin JH. Inhibition of prothrombinase by human secretory phospholipase A2 involves binding to factor Xa. J Biol Chem. 1998;273:23764-23772

26. Boerger LM, Morris PC, Thurnau GR, Esmon CT, Comp PC. Oral contraceptives and gender affect protein S status. Blood. 1987;69:692-694
27. Schwalbe RA, Dahlbäck B, Nelsestuen GL. Independent association of serum amyloid P component, protein S, and complement C4b with complement C4b-binding protein and subsequent association of the complex with membranes. J Biol Chem. 1990;265:21749-21757
28. Lu D, Xie R, Rydzewski A, Long G. The effect of N-linked glycosylation on molecular weight, thrombin cleavage, and functional activity of human protein S. Thromb Haemost. 1997;77:1156-1163.
29. Rosing J, Bakker HM, Thomassen MC, Hemker HC, Tans G. Characterization of two forms of human factor Va with different cofactor activities. J Biol Chem. 1993;268:21130-21136
30. Ortel TL, Quinn-Allen MA, Keller FG, Peterson JA, Larocca D, Kane WH. Localization of functionally important epitopes within the second C-type domain of coagulation factor V using recombinant chimeras. J Biol Chem. 1994;269:15898-15905
31. Fernandez JA, Hackeng TM, Kojima K, Griffin JH. The carbohydrate moiety of factor V modulates inactivation by activated protein C. Blood. 1997;89:4348-4354
32. Koenen RR, Gomes L, Tans G, Rosing J, Hackeng TM. The Ser460Pro mutation in recombinant protein S Heerlen does not affect its APC-cofactor and APC-independent anticoagulant activities. Thromb Haemost. 2004;In press
33. Giri TK, Yamazaki T, Sala N, Dahlback B, de Frutos PG. Deficient APC-cofactor activity of protein S Heerlen in degradation of factor Va Leiden: a possible mechanism of synergism between thrombophilic risk factors. Blood. 2000;96:523-531
34. Morboeuf O, Borgel D, Aiach M, Kaabache T, Gandrille S, Gaussem P. Expression and characterization of recombinant protein S with the Ser 460 Pro mutation. Thromb Res. 2000;100:81-88
35. Giri TK, Linse S, Garcia de Frutos P, Yamazaki T, Villoutreix BO, Dahlback B. Structural requirements of anticoagulant protein S for its binding to the complement regulator C4b-binding protein. J Biol Chem. 2002;277:15099-15106

Chapter 5

The APC-independent anticoagulant activity of protein S in plasma is decreased by elevated prothrombin levels due to the prothrombin G20210A mutation.

Koenen RR, Tans G, van Oerle R, Hamulyak K, Rosing J and Hackeng TM.

Blood, 2003, 102:1686-1692

Abstract

Protein S exhibits anticoagulant activity independent of activated protein C (APC). An automated factor Xa-based one-stage clotting assay was developed which enables quantification of the APC-independent activity of protein S in plasma from the ratio of clotting times (protein S Ratio: pSR) determined in the absence and presence of neutralizing antibodies against protein S. The pSR was 1.62 ± 0.16 (mean ± SD) in a normal population (n=60), independent of the plasma levels of factors V, VIII, IX, X, protein C, antithrombin, and not affected by the presence of factor V_{Leiden}. The pSR strongly correlates with the plasma level of protein S and is modulated by the plasma prothrombin concentration. In a group of 16 heterozygous protein S-deficient patients, the observed mean pSR (1.31 ± 0.09) was significantly lower than the mean pSR of the normal population (1.62 ± 0.16). The pSR of plasma from carriers of the prothrombin G20210A mutation (n = 46) was significantly lower (1.47 ± 0.21) than the pSR of the 60 controls (1.62 ± 0.16). We propose that the decreased APC-independent anticoagulant activity of protein S in plasma with elevated prothrombin levels may contribute to the thrombotic risk associated with the prothrombin G20210A mutation.

Introduction

Protein S is a vitamin K-dependent anticoagulant protein that plays an important role in the regulation of blood coagulation. Several studies have indicated that a deficiency in protein S is a risk factor for venous thrombosis [1-3]. Protein S regulates blood coagulation by various mechanisms. Protein S acts as a cofactor for activated protein C (APC) in the inactivation of factor Va [4,5] and VIIIa [6,7], and it exhibits anticoagulant activity in the absence of APC [8-11] i.e. protein S was shown to directly inhibit the activity of the prothrombin and intrinsic factor X-activating complexes on negatively charged phospholipids, platelets and endothelial cells[10-14]. In plasma approximately 60% of protein S circulates in complex with C4b binding protein (C4BP), a regulator of the classical complement cascade [15].

Binding of protein S to C4BP completely inhibits the APC-cofactor activity of protein S [16], but the complex still exhibits APC-independent anticoagulant activity [10]. Protein S-binding to phospholipids and direct interactions with factors Va and Xa appear to be important for its inhibitory action [10,11,13]. However, the precise molecular mechanisms by which protein S exerts APC-independent anticoagulant activity still remain unknown.

It was previously shown that purified protein S preparations contain two fractions, a multimeric fraction having a (unnatural) very high affinity for phospholipids and a normal (natural) fraction[17]. Prothrombinase inhibition by the isolated multimeric fraction has been observed to be strongly phospholipid dependent. These protein S multimers, although present in percentage amounts, can make up for >90% of the total inhibitory potential of purified protein S, depending on the phospholipid concentration. Because protein S multimers are absent in plasma[17], the anticoagulant activity of protein S in plasma in the absence of APC can therefore be regarded as the natural activity not obscured by the in vitro generated multimers. These observations emphasize the importance of studying unmodified protein S in its natural environment, i.e. full plasma.

Several genetic defects are known that influence plasma protein S levels [18], APC-cofactor activity of protein S [19], or both[20,21]. However, no functional abnormalities have been described for the APC-independent activity of protein S. This may in part be due to the fact that it is difficult to measure the APC-independent activity of protein S in plasma. An assay for the APC-independent activity was developed in which coagulation in plasma was triggered with diluted tissue factor in the presence of low molecular weight heparin (LMWH) [22]. The ratio of clotting times measured in plasma in the absence and presence of a functional inhibitory antibody against protein S was used as a measure of the APC-independent activity of protein S. However, due to the presence of heparin and because plasma samples were diluted 1:1 with protein S-depleted plasma, the reaction conditions chosen in the LMWH-based assay [22] may mask the influence of other plasma components on the APC-independent activity of protein S.

In the present paper we have investigated the APC-independent anticoagulant activity of protein S in undiluted plasma without the use of heparin.

Materials and methods

Protein S and factor Xa were purchased from Enzyme Research Laboratories (South Bend, IN). HEPES was from ICN (Costa Mesa, CA). Bovine serum albumin (BSA, initial fractionation by heat shock) was purchased from Sigma (St. Louis, MO). Fragmin™ (Dalteparin sodium) was obtained from Pharmacia and Upjohn (Woerden, The Netherlands). Recombinant human tissue factor (Innovin™) was from Dade Behring (Marburg, Germany). Cyanogen bromide (CNBr)-activated Sepharose, QAE-Sephadex and Mono Q resins were purchased from Pharmacia (Uppsala, Sweden). 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS) were obtained from Avanti Polar Lipids (Alabaster, AL). Protein C-depleted plasma and its corresponding parent plasma as well as factor II, V, VII, VIII, IX, X and antithrombin-depleted plasma were purchased from Affinity Biologicals (Hamilton, Ontario, Canada). Antibodies against protein S were obtained from DAKO (Glostrup, Denmark). The REAADS Monoclonal Free Protein S Antigen Test Kit was purchased from Corgenix (Westminster, CO). Ecarin™ was obtained from Pentapharm (Basel, Switzerland). Other reagents were of the highest grade available. Human prothrombin was purified as previously described [7].

Specificity of polyclonal antibodies against protein S

The polyclonal antibody against protein S was specific for protein S as determined in an ELISA using normal and protein S deficient plasma. The polyclonal antibody was tested on other vitamin K-dependent coagulation factors (II, X, protein C) in an ELISA setup and cross-reactivity was absent. On SDS-PAGE Western Blots from plasma and purified protein S only one band was visible when the polyclonal antibody against protein S was used. When tested on purified prothrombin on native and SDS-PAGE Western Blots, no signal was obtained.

Protein S-depleted plasma incubated with and without antibodies showed identical turbidity change (clotting) curves, indicating that the antibody against protein S does not functionally affect plasma coagulation proteins other than protein S.

Collection of plasma samples

After discarding the first 2 ml, nine parts of venous blood from 80 healthy hospital workers, post-thrombotic patients with protein S deficiency (n = 16) and prothrombin G20210A carriers (n=46, 15 men and 31 women) were drawn from the antecubital vein via an open system and collected in one part of 3.8 % (0.13 M) trisodium citrate. The blood was centrifuged at 3000xg to separate plasma from blood cells and centrifuged again at 19000xg to obtain platelet poor plasma. Normal pooled plasma was prepared by pooling plasma from 80 healthy individuals (51 men and 29 women; mean age 40 yr). Individual normal plasma samples were from the last 60 individuals (40 men and 20 women; mean age 40 yr) who donated blood for the normal pool. The presence of factor V_{Leiden} was determined using the prothrombinase-based APC resistance assay as previously described [23], and via a PCR-based method. None of the volunteers that participated in this study was using oral anticoagulants and/or oral contraceptives.

Preparation of depleted plasmas

Fresh venous blood was drawn via an open system from nine healthy and sober volunteers (6 men, 3 women) as described above. Protein S-depleted plasma was obtained by incubating the pooled plasma batch-wise with rabbit polyclonal anti-protein S antibodies coupled to Sepharose CL-4B for 3 hours at room temperature after which the resin was removed by centrifugation. Before use, both resins had been equilibrated in 25 mM Hepes pH 7.7, 175 mM NaCl (HEPES-buffered saline: HBS). As a control, the parent plasma was incubated with Sepharose CL-4B. The protein S-total antigen levels of the plasmas were determined by ELISA and were 85 % of that of normal pooled plasma in the case of parent plasma and ~1% in the case of the protein S-depleted plasma.

Preparation of hemolytic and lipemic plasma

Fresh venous blood was drawn via an open system from a healthy male volunteer after overnight fasting as described above. The anticoagulated blood was divided in two portions. One portion was given an ultrasonic pulse of approximately 0.5 s using a probe tip sonicator (set at 7 μm peak to peak amplitude). Platelet poor plasma was prepared from both plasmas as described above. The sonicated plasma was red due to hemolysis by sonication. Several hours later, the same volunteer was offered a meal consisting of chicken livers and French fries and blood was taken 2 hours after the meal. Platelet-poor lipemic plasma was prepared from the blood as described above.

Assays for the APC-independent anticoagulant activity of protein S

Heparin-based assay performed on microtiter plates. The heparin-based assay for the APC-independent anticoagulant activity of protein S was performed as described [22]. Briefly, to 75 μl plasma (37.5 μl subject plasma and 37.5 μl protein S-depleted plasma) in a 96 wells microtiter plate, low molecular weight heparin (Fragmin™) was added to a final concentration of 1 anti-factor Xa U/ml. The plasma was incubated in the presence of 9 μl DAKO rabbit polyclonal anti-protein S antibodies or buffer for 30 minutes at room temperature. Coagulation was initiated by addition of 25 μl human recombinant tissue factor (Innovin™) diluted 1:200 in HBS containing 68 mM $CaCl_2$ and 5 mg/ml BSA. The coagulation process was monitored at 37 °C in a plate reader at 405 nm. The clotting time was defined as the time required to obtain half-maximal absorbance change at 405 nm.

The factor Xa-based assay performed on microtiter plates. Plasma (100 μl) was incubated in the presence of 12 μl DAKO rabbit polyclonal anti-protein S antibodies (2.5 μM final concentration) or buffer for 30 minutes at room temperature. In a 96-wells microtiter plate, 50 μl plasma was incubated for 3 minutes at 37 °C and coagulation was triggered by the addition of 150 μl of 25 pM factor Xa, 8 μM DOPS/DOPC (20/80, M/M) vesicles in HBS containing 11 mM $CaCl_2$ and 5% BSA.

This resulted in final concentrations of 20 pM factor Xa, 6 μM DOPS/DOPC (20/80, M/M) vesicles, 5 mM $CaCl_2$ and 5% (w/v) BSA. Progress of coagulation was monitored at 37 °C in a plate reader at 405 nm. The clotting time was defined as the time required to obtain half-maximal absorbance change at 405 nm. The APC-independent activity of protein S in normal pooled plasma was completely inhibited at an anti-protein S antibody concentration > 2.25 μM (data not shown).

Automated factor Xa-based assay. The Xa-based assay was adapted to be performed on an ACL-300 (Instrumentation Laboratory, Milan, Italy) with the following coagulation program parameters: sample position: 50 μl, reagent position 1: 150 μl, incubation time: 210 sec, int. ramp time: 1 sec, delay: 0 sec, acquisition time: 600 sec and rotation speed: 1200 rpm. A PT-FIB reagent container (Instrumentation Laboratory) was used at position 1 for the start reagent containing 25 pM factor Xa, 8 μM DOPS/DOPC (20/80, M/M) vesicles, 11 mM $CaCl_2$ and 5% BSA in HBS. The clotting time was defined as the time needed for the absorbance to pass a threshold value of 0.06 OD units above the initial absorbance of the sample (onset of coagulation).

Preparation of phospholipid vesicles

Phospholipid vesicles were prepared in glass test tubes by evaporating *400 μl of a 10 mM DOPS/DOPC (20/80, M/M) stock solution in* chloroform/methanol under nitrogen flow. The resulting phospholipid film was suspended in 2 ml of HBS and sonicated on ice to clarity using a probe tip sonicator (set at 7.5 μm peak to peak amplitude). The resulting 2 mM DOPS/DOPC (20/80, M/M) vesicle suspension was diluted tenfold and aliquots were stored under nitrogen at -80 °C.

Purification of human C4BP containing the β-chain

The protein S-C4BP complex was isolated from 20 units of freshly frozen plasma (5 L) as previously described [7]. Briefly, the plasma was subjected to barium citrate, ammonium sulfate precipitation and QAE-Sephadex anion exchange chromatography. The protein S-C4BP complex was obtained as an early peak eluting from the QAE-Sephadex column.

The pooled fraction containing the protein S-C4BP complex was dialyzed overnight against 25 mM Tris-HCl pH=7.4, 150 mM NaCl, 2 mM EDTA and loaded onto Mono Q resin (1 mL bed volume). A linear gradient of 25 mM Tris-HCl pH=7.4, 600 mM NaCl, 2 mM EDTA was applied and free C4BP was collected as a peak at approximately 250 mM NaCl. The C4BP obtained via this method contained approximately 5% (m/m) protein S.

Prothrombin assay

The concentration of prothrombin was determined using the purified prothrombin activator Ecarin™ from the venom of the saw-scaled viper (*Echis carinatus*) as described before [24].

Determination of total and free protein S antigen

Total protein S antigen was determined using an ELISA with rabbit-polyclonal antibodies (DAKO) against human protein S and was performed essentially as described [17], except that diluted plasma samples were incubated in the microtiter plate overnight at room temperature instead of 1 hour at room temperature to facilitate C4BP-protein S complex dissociation. Free protein S antigen was determined using a monoclonal free protein S antigen determination kit according to the manufacturer's instructions. Samples were processed as described [25]. All antigen determinations were performed using normal pooled plasma as the standard and antigen levels were expressed as percentage of that present in normal pooled plasma.

Statistical analysis

Population means were compared using a t-test. Correlations given in this report are represented as Pearson correlation coefficients.

Results

Comparison of factor Xa and heparin-based assays for the APC-independent anticoagulant activity of protein S.

In the reported assay for the measurement of APC-independent anticoagulant activity of protein S [22], plasma samples are diluted 1:1 in protein S-depleted plasma, and low molecular weight heparin (LMWH) is added to enhance the inhibition of coagulation by protein S. Although these assay conditions appeared to be necessary to effectively measure the anticoagulant effect of protein S, the addition of LMWH changes the milieu in which protein S normally acts and therefore may cause incorrect assessment of its in vivo activity.

We have investigated the possibility to measure the APC-independent activity of protein S in undiluted plasma in the absence of LMWH. In the test that we have developed, coagulation was initiated by the addition of factor Xa, phospholipids and $CaCl_2$, in plasma preincubated with and without inhibitory polyclonal antibodies against protein S. Clotting was monitored by following the turbidity change at 405 nm. Due to inhibition of the anticoagulant action of protein S by the anti-protein S antibodies, the lag-time that precedes clotting of plasma was shorter in antibody-treated plasma than in untreated plasma (Fig. 1A). The protein S-dependent anticoagulant ratio (PSdAR), calculated from the time required to obtain the half-maximal turbidity change in the absence and presence of antibody, was approximately 1.8. To compare the performance of our test to that of the reported assay [22], normal pooled plasma was diluted 1:1 in protein S-depleted plasma in the presence of LMWH and coagulation was initiated with diluted tissue factor in plasma with and without anti-protein S antibody, as described [22]. Again, plasma incubated with anti-protein S antibodies had a shorter clotting time than untreated plasma (Fig. 1B). However, comparison of figure 1A and 1B shows that the change in turbidity proceeded much slower in the LMWH-based assay than in the factor Xa-based assay. In order to accurately determine the PSdAR, coagulation must be allowed to reach an absolute endpoint, which in the LMWH-based assay took 1.5–2 hours.

For both assays the relation between assay duration (i.e. the time taken as endpoint of fibrin formation) and the PSdAR was investigated. The PSdAR of the factor Xa-based assay was virtually independent of the time at which the endpoint was taken, whereas in the LMWH-based assay the ratio varied significantly with the endpoint time (Fig. 1C).

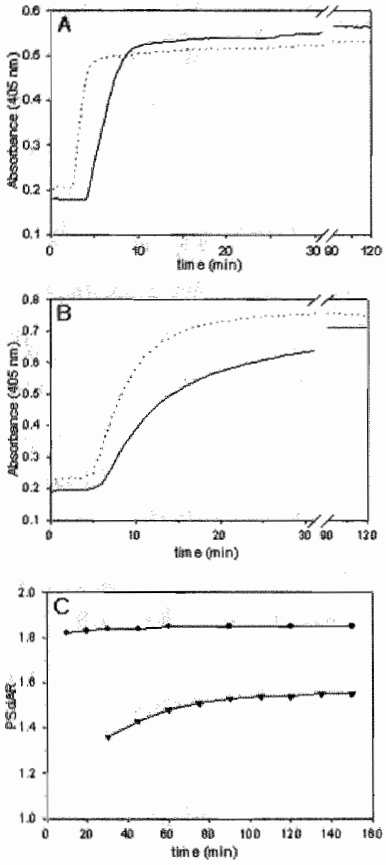

Figure 1. Factor Xa and LMWH-based assays for the APC-independent activity of protein S in plasma.
Normal pooled plasma was incubated for 30 minutes with (dashed line) or without (solid line) anti-protein S neutralizing antibodies. (A) Coagulation was triggered with $CaCl_2$ (5 mM), factor Xa (20 pM) and phospholipids (6 μM DOPS/DOPC, 20/80 M/M) at indicated final concentrations. (B) LMWH was added and coagulation was triggered with diluted tissue factor (Innovin™) as described in the Methods section. Fibrin formation was followed by measuring the change in turbidity at 405 nm. (C) Half-maximal value clotting time ratios (PSdAR) of the factor Xa-based assay (filled circles) and LMWH-based assay (filled triangles) are represented as a function of assay duration.

Determination of the APC-independent anticoagulant activity of protein S from the onset of coagulation.

Visual inspection of the clotting curves (Fig. 1A,B) indicates that, particularly in the factor Xa-based assay there is a distinct difference between the lag times that precede the onset of absorbance change in antibody-treated and untreated plasma (Fig. 1A).

This prompted us to investigate whether this clotting lag time ratio (protein S Ratio, pSR: the ratio of the times required to reach an absorbance change of 0.06 mOD above base-level in untreated plasma and antibody-treated) could be used as an alternative for the PSdAR. A high correlation between pSR and PSdAR was observed (r^2 = 0.998) when the amount of protein S present in plasma was varied by mixing varying amounts of protein S-depleted plasma and parent plasma (Fig. 2). When the pSR is used to measure the APC-independent anticoagulant activity of protein S, the assay is well suited to be performed on an ACL-300 auto-analyzer.

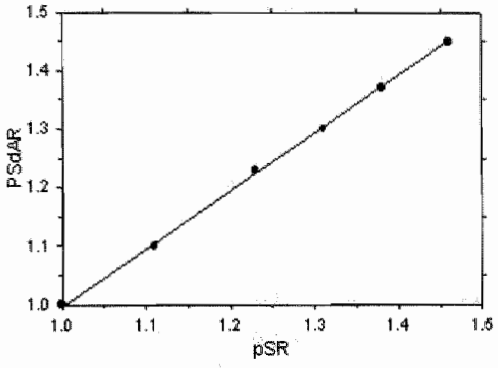

Figure 2. Relation between the PSdAR and the pSR.
Protein S-depleted plasma was mixed with increasing amounts of its corresponding parent plasma (0, 20, 40, 60, 80 and 100 % parent plasma) and coagulation was triggered with $CaCl_2$ (5 mM), factor Xa (20 pM) and phospholipids (6 μM DOPS/DOPC, 20/80 M/M) at indicated final concentrations. The PSdAR was plotted against the pSR.

Effect of factor Xa and phospholipid concentrations on the protein S Ratio (pSR).

The influence of the amounts of factor Xa and phospholipid on the pSR was investigated by activating coagulation with various concentrations of factor Xa at a constant phospholipid concentration. At 6 μM phospholipid (DOPS/DOPC, 20/80, M/M) the pSR depended on the factor Xa concentration and increased with decreasing amounts of factor Xa (Fig. 3A).

Remarkably, at 20 pM of factor Xa used (final concentration), the pSR was only slightly dependent of phospholipid concentration (Fig. 3B). This is in contrast with a strong phospholipid-dependence of the APC-independent activity of protein S observed in purified systems[14], which most likely results from in vitro multimerization effects due to protein purification methods[17]. Final concentrations chosen for the assay were 20 pM factor Xa and 6 μM DOPS/DOPC (20/80, M/M). Under these conditions the pSR could be sensitively measured while inter-assay variations remained within an acceptable low range (see below).

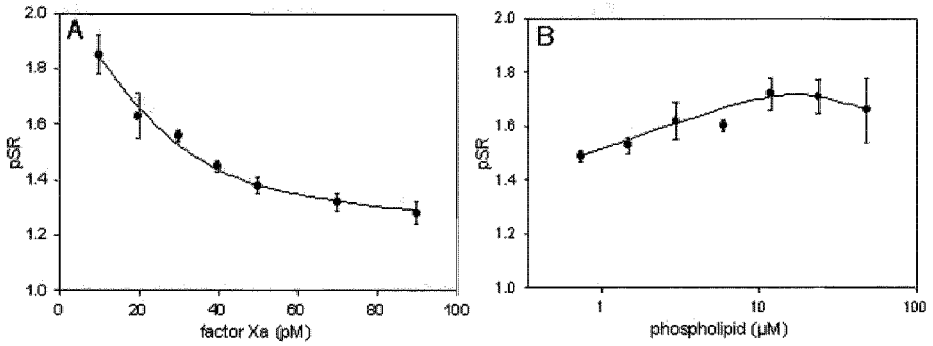

Figure 3. Effect of the factor Xa and phospholipid concentration on the pSR. Normal pooled plasma was incubated for 30 minutes with and without antibodies against protein S. Coagulation was triggered with 5 mM $CaCl_2$ and (A) 6 μM phospholipid vesicles (DOPS/DOPC, 20/80, M/M) and 10, 20, 30, 40, 50, 70 and 90 pM factor Xa or (B) 20 pM factor Xa and 0.75, 1.5, 3, 6, 12, 24 and 48 μM phospholipid vesicles (DOPS/DOPC, 20/80, M/M). The pSR is indicated as a function of (A) the factor Xa or (B) the phospholipid concentration. Mean ± SD of three measurements are shown.

A calibration curve made by mixing protein S-depleted plasma with its corresponding parent plasma (Fig. 4), showed that the pSR was linearly dependent on the amount of parent plasma and varied between 1.04 ± 0.02 (protein S-depleted plasma) and 1.46 ± 0.05 (parent plasma). The pSR of the parent plasma (1.46) was lower than that of normal pooled plasma (1.63) because the plasma was diluted (to 85%) during the batch wise depletion procedure (incubation with Sepharose CL-4B with or without anti-protein S antibodies).

Incubation of normal pooled plasma with 200 nM of purified C4BP resulted in a minor decrease of the pSR by 11% from 1.59 to 1.41 confirming that the complex of protein S and C4BP still has APC-independent activity[10].

Repeated determination showed that the pSR of normal pooled plasma was 1.63 ± 0.08 (mean ± SD, 57 measurements) yielding an inter-assay variance of 4.7%. The intra-assay variance of the pSR was 1.6 % (9 measurements). The pSR was not significantly influenced by hemolysis (sonication) or high lipid content of plasma due to high-fat diet (data not shown).

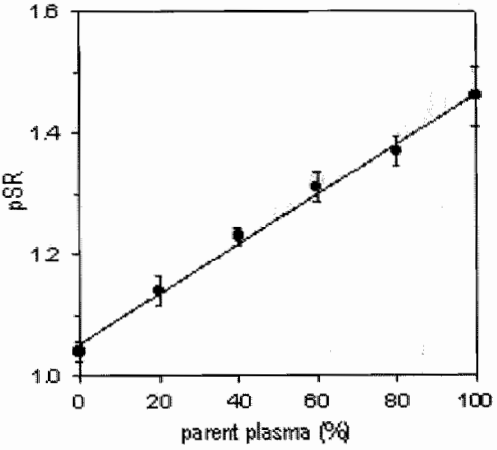

Figure 4. pSR as a function of total protein S in plasma.
Protein S-depleted plasma was mixed with parent plasma and the APC-independent activity of protein S (pSR) was determined with the factor Xa-based assay. pSRs are plotted as a function of percentage of parent plasma. Each data point represents the mean value ± SD of at least three measurements.

The effect of plasma coagulation factor levels on the APC-independent anticoagulant activity of protein S.

The influence of variation of the plasma levels of the coagulation proteins prothrombin, factors V, VII, VIII, IX, X and antithrombin was investigated by mixing plasma depleted of the respective coagulation protein with normal pooled plasma and determining the pSR. The pSR appeared only to be dependent on the prothrombin level in plasma and was increased almost twofold at a prothrombin level of 33% (Fig. 5).

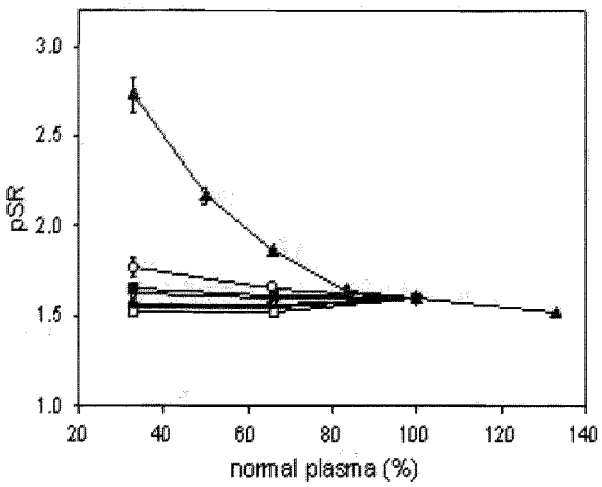

Figure 5. Influence of plasma coagulation proteins on the pSR.
pSRs were plotted as a function of the concentration of prothrombin (filled triangles), factor V (filled circles), factor VII (open circles), factor VIII (inverted filled triangles), factor IX (inverted open triangles), factor X (filled squares) or antithrombin (open squares). Coagulation factor-depleted plasmas were mixed with normal pooled plasma. Each data point represents the mean ± SD of at least three measurements. Purified human prothrombin was added to normal pooled plasma in order to obtain plasma with 133% prothrombin.

In order to exclude involvement of protein C and APC in the assay, the pSR of protein C-depleted plasma was compared with the pSR of its corresponding parent plasma. The pSR of protein C-depleted plasma (1.77 ± 0.02) was even slightly higher than that of its corresponding parent plasma (1.69 ± 0.01) while protein S levels were identical, indicating that the assay outcome is independent of (activated) protein C levels in plasma. In addition, polyclonal antibodies against protein C that completely inhibit APC-anticoagulant activity were applied in the current test. Polyclonal antibodies against protein C did not affect the clotting times in the assay, either in the absence or presence of antibodies against protein S, yielding identical pSR values (1.66) when compared to the same plasma without antibodies against protein C. Therefore, the traces of APC that were reported to exist in blood in vivo[26] are likely inhibited by serpins in plasma samples used in our study.

Furthermore, the contribution of in situ activation of protein C during our assay duration is considered absent or too low to affect the clotting times and thus the pSR.

The effect of plasma protein S and prothrombin levels on the pSR.

The APC-independent activity of protein S and the plasma levels of protein S (total and free) and prothrombin were measured in plasma of 60 of the 80 healthy individuals who contributed to the normal plasma pool, in plasma from 16 heterozygous protein S deficient individuals and in 46 carriers of the prothrombin G20210A mutation (Table 1, Fig. 6). The pSR of factor V_{Leiden} carriers are indicated by open circles (Fig. 6).

pSR values of the normal population were distributed symmetrically around an average of 1.62 ± 0.16 (which is very close to the pSR (1.63 ± 0.08) of normal pooled plasma) with values ranging between 1.35 and 2.24 (Fig. 6).

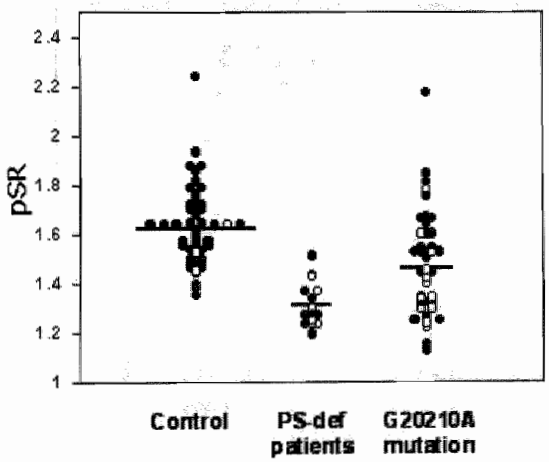

Figure 6. APC-independent anticoagulant activity of protein S in plasma from healthy controls, protein S-deficient patients and carriers of the prothrombin G20210A mutation.
The APC-independent anticoagulant activity of protein S (pSR) was determined with the factor Xa-based assay in plasma from 60 healthy controls, 16 protein S-deficient patients and 46 prothrombin G20210A carriers as described in the method section. Factor V_{Leiden} carriers are indicated by open circles. The mean value, which is represented as a horizontal bar, was 1.62 for the control population, 1.31 for the protein S-deficient patients and 1.47 for the prothrombin G20210A carriers.

The pSR correlated well with protein S levels and this correlation was stronger with total protein S (Fig. 7, r=0.520, P<0.001) than with free protein S (data not shown, r=0.365, P=0.004). Considering the influence of prothrombin on the pSR (Fig. 5), the prothrombin levels in the control population were measured. The average prothrombin level of the control population was 99 ± 13.0 % (Table 1) which together with the protein S-total and free levels was within normal range (Table 1) and the pSR did not significantly correlate with the plasma prothrombin concentration (r=-0.168, P=0.20). However, in a regression model both variables were indicated as determinants of the pSR (r=0.566, P<0.001) with protein S having an upregulating effect and prothrombin having a downregulating effect on the pSR.

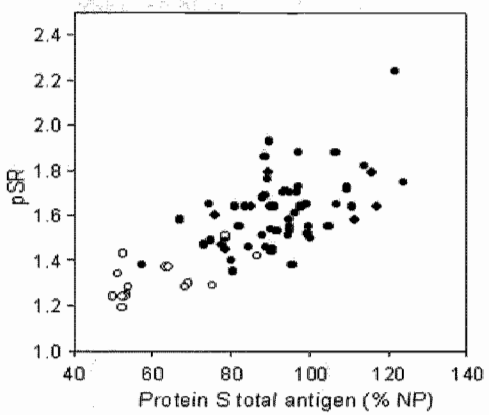

Figure 7. Correlation between APC-independent anticoagulant activity of protein S and plasma levels of protein S-total antigen.
The APC-independent anticoagulant activity of protein S (pSR) was determined with the factor Xa-based assay in plasma from 60 healthy controls (filled circles) and 16 protein S-deficient patients (open circles).

In the plasmas of 16 individuals with protein S deficiency the mean pSR (1.31 ± 0.09) (Table 1) was significantly lower (P<0.001) than the pSR of the normal population (Fig. 6). This observation was in agreement with the decreased protein S levels but normal prothrombin levels in the protein S-deficient patients (Table 1). Unlike free protein S (r=0.420, p=0.105), only total protein S was found to correlate significantly with the pSR in the protein S-deficient patients (r=0.586, p<0.05).

Interestingly, there was a significant negative correlation between prothrombin levels and the pSR in protein S-deficient patients ($r=-0.605$, $P<0.05$). This indicates that the effect of prothrombin on the pSR is increased at low protein S concentrations.

To identify a modulatory effect of increased prothrombin levels associated with the prothrombin G20210A mutation on the APC-independent activity of protein S, the pSR of a group of 46 prothrombin G20210A carriers was determined (Fig. 6). The average pSR of the prothrombin G20210A carriers was 1.47 ± 0.21, which was significantly lower ($P<0.001$) than the mean pSR of the control group (1.62, Table 1). The prothrombin levels of the prothrombin G20210A carriers were significantly elevated (Table 1) whereas protein S-total levels were similar to the protein S-total levels of the control group. Protein S antigen values in men are significantly higher than in women. Since 40/60 of the control population and 15/46 of the prothrombin mutant carriers were men, it was important to know if the results would change if only men or only women were considered. After stratification, a significant difference remained between the pSR of the control and prothrombin G20210A populations in men and women, respectively ($p<0.05$). Taken together, increased prothrombin levels can explain the decreased APC-independent activity of protein S observed in carriers of the prothrombin G20210A mutation.

Table 1: Total and free protein S antigen levels, prothrombin levels and APC-independent activity of protein S (pSR)

Population	Total protein S (% NP)	Free protein S (% NP)	Prothrombin (% NP)	pSR
Control	93 ± 13	101 ± 18	99 ± 13	1.62 ± 0.16
Protein S-deficiency	61 ± 11**	47 ± 25**	95 ± 14	1.31 ± 0.09**
Prothrombin G20210A	99 ± 22	91 ± 25*	123 ± 24**	1.47 ± 0.21**

Means ± standard deviation are shown.

* $P<0.05$ compared to control population

** $P<0.001$ compared to control population

Discussion

In this paper we have described an automated one-stage coagulation assay that enables quantification of the APC-independent anticoagulant activity of protein S in plasma. In this assay, coagulation is initiated in plasma with low amounts of factor Xa, phospholipids, and $CaCl_2$, and the time to onset of coagulation is compared with that of plasma in which protein S is inhibited by antibodies. Using this assay, the APC-independent activity of protein S could be effectively measured without the necessity of adding coagulation inhibitors like heparin or diluting plasma in protein S-depleted plasma as was reported previously [22].

In the present assay a clotting time ratio (pSR), calculated from coagulation lag-times, was used to quantify the APC-independent activity of protein S. This is in contrast to the ratio of times required to reach half-maximal turbidity during the clotting of plasma, used in the previously reported heparin-based assay (PSdAR [22]). While the present assay has the advantage that clotting time ratios can be determined from the lag times within 5 minutes, stable turbidity endpoint determinations in the heparin-based assay are only reached at approximately 60 minutes after initiation of coagulation. In the heparin-based assay pSR measurements (i.e. ratios calculated from clotting lag times) are not possible due to very small differences between the lag times of anti-protein S antibody-treated plasma and non-treated plasma. The present pSR assay offers the advantage of quick measurements (< 5 min) and it enables determination of the pSR on auto-analyzers.

The pSR assay was set up to directly determine the APC-independent anticoagulant activity of protein S without dilution of the test plasma in protein S-depleted plasma. Therefore, plasma components that might modulate the APC-independent activity of protein S are more likely to be unveiled. The APC-independent activity of protein S (pSR) was 1.63 in normal pooled plasma. The pSR was independent of plasma concentrations of factors V, VIII, IX and X and was not affected by the presence of the factor V_{Leiden} mutation. Factor VII and antithrombin have only a minor influence on the pSR, and a possible effect of (activated) protein C was excluded.

Low levels of antithrombin (33%) resulted in a slightly decreased pSR (1.53 ± 0.01) and the pSR was slightly elevated at low factor VII levels (pSR = 1.77 ± 0.05 at 33% factor VII), phenomena for which we have as yet no explanation. Prothrombin appeared to have a large effect on the APC-independent anticoagulant activity of protein S. The pSR increased from 1.52 ± 0.01 at 133% prothrombin to 2.73 ± 0.10 at 33% prothrombin. The enhancement of APC-independent activity of protein S at low prothrombin concentrations is most likely explained by a decreased competition between prothrombin and protein S for binding to the prothrombinase complex components (factor Xa, factor Va and phospholipid).

Modulation of protein S by prothrombin has already been observed in previous studies. It was reported that protein S was able to directly inhibit factor Xa in the absence of factor Va, and this inhibition could be counteracted by increasing amounts of prothrombin [8]. Later it was showed that binding of prothrombin to immobilized factor Va was effectively inhibited by protein S [9]. Interestingly, it was also shown that prothrombin was able to inhibit the APC-cofactor activity of protein S [27] and inhibition of APC-cofactor function as well as APC-independent activity of protein S by prothrombin may, at least in part, share a mechanistic basis. However, structural requirements for the APC-cofactor and APC-independent anticoagulant activities of protein S have been shown to be different [28]. In addition, prothrombin has been shown to inhibit APC activity in the absence of protein S [29] and therefore establishment of the mechanism by which protein S inhibits prothrombinase will not be straightforward.

We only observed a modest effect of phospholipid concentration on the APC-independent activity of protein S in our assay. We believe that an effect of the phospholipid concentration on APC-independent activity of protein S is particularly observed when purified protein S preparations are used in reconstituted systems. It was reported that purified protein S preparations contain multimers as a result from in vitro purification procedures[17]. These protein S multimers have a high phospholipid-binding affinity (KD< 1 nM).

A 100-fold increased APC-independent anticoagulant activity was found due to the ability of multimers to inhibit phospholipid-dependent coagulation reactions[17]. The overall APC-independent activity of a protein S preparation is therefore governed by this small percentage of protein S multimers that act via a different mechanism (direct competition for phospholipid binding sites) and that explains previously reported results in in vitro reaction systems[13],[14]. The fact that protein S multimers are absent in normal plasma[17], explains why the APC-independent activity of protein S in plasma is independent on the phospholipid concentration i.e. the mechanism of competition of phospholipid surface is not the native mechanism by which protein S attenuates blood coagulation in absence of APC. The in vitro multimerization of purified protein S emphasized the need to study the APC-independent activity of native protein S in its native environment.

The pSR of normal pooled plasma (1.63 ± 0.08) corresponded well with the mean pSR of the normal individuals (1.62 ± 0.16). However, there was considerable variation of the pSR within the normal population hinting at individual differences. The pSR correlated both with the free and total plasma levels protein S. The better correlation of the pSR with total protein S than with free protein S supports the observation that the protein S-C4BP complex as well as free protein S contribute to the APC-independent inhibition of the prothrombinase complex [10]. In the present assay this was confirmed by a small decrease in pSR after all protein S was complexed to exogenously added C4BP.

Considering the strong dependence of the pSR on plasma protein S levels it is not surprising that in a group of protein S-deficient patients the pSR was significantly lower than that of the normal population. At lower levels of protein S, factors other than protein S (e.g. prothrombin) may contribute to the assay outcome. This idea is supported by the observation that a significant negative correlation was found of the pSR with plasma prothrombin levels ($r=-0.605$, $P<0.05$) in protein S-deficient patients. In the normal population however, a poor correlation between prothrombin and the pSR was observed.

This can be explained by opposite effects on the pSR by prothrombin and protein S, of which the plasma levels are linked by their synthesis in the liver. We therefore subsequently performed a multivariate regression model in which prothrombin and protein S-total and free levels were added as parameters. This model revealed prothrombin and protein S-total as significant determinants of the pSR in plasma (data not shown).

Analogous to the uncoupling of protein S and prothrombin levels in the group of heterozygous protein S-deficient patients, increased plasma prothrombin levels may decrease the APC-independent activity of protein S in plasma when the prothrombin level in plasma changes independent of protein S. Indeed, carriers of prothrombin G20210A who have a mutation in the 3' untranslated region of the prothrombin gene resulting in elevated plasma prothrombin[30] (Table 1), were observed to have a significantly lower pSR (1.47 ± 0.21, $P<0.001$) than the control group (1.62 ± 0.16) while total protein S levels were not significantly different. These observations may, at least in part, provide a possible rationale for the thrombotic risk associated with the prothrombin G20210A mutation[30]

In conclusion, the assay presented in this report enables reproducible and efficient measurement of APC-independent activity of protein S in plasma and will contribute to a better understanding of the mechanism by which protein S attenuates blood coagulation. Measuring APC-independent anticoagulant activity of protein S in plasma might offer an additional tool to establish the physiological importance of the anticoagulant activity of protein S in the absence of APC.

References

1. Comp PC, Nixon RR, Cooper MR, Esmon CT. Familial protein S deficiency is associated with recurrent thrombosis. J Clin Invest. 1984;74:2082-2088
2. Comp PC, Esmon CT. Recurrent venous thromboembolism in patients with a partial deficiency of protein S. N Engl J Med. 1984;311:1525-1528
3. Faioni E, Valsecchi C, Palla A, Taioli E, Razzari C, Mannucci P. Free protein S deficiency is a risk factor for venous thrombosis. Thromb Haemost. 1997;78:1343-1346.
4. Walker FJ. Regulation of activated protein C by protein S. The role of phospholipid in factor Va inactivation. J Biol Chem. 1981;256:11128-11131
5. Rosing J, Hoekema L, Nicolaes GA, Thomassen MC, Hemker HC, Varadi K, Schwarz HP, Tans G. Effects of protein S and factor Xa on peptide bond cleavages during inactivation of factor Va and factor VaR506Q by activated protein C. J Biol Chem. 1995;270:27852-27858
6. Walker FJ, Chavin SI, Fay PJ. Inactivation of factor VIII by activated protein C and protein S. Arch Biochem Biophys. 1987;252:322-328
7. Koedam JA, Meijers JC, Sixma JJ, Bouma BN. Inactivation of human factor VIII by activated protein C. Cofactor activity of protein S and protective effect of von Willebrand factor. J Clin Invest. 1988;82:1236-1243
8. Mitchell C, Kelemen S, Salem H. The anticoagulant properties of a modified form of protein S. Thromb Haemost. 1988;60:298-304.
9. Heeb MJ, Mesters RM, Tans G, Rosing J, Griffin JH. Binding of protein S to factor Va associated with inhibition of prothrombinase that is independent of activated protein C. J Biol Chem. 1993;268:2872-2877
10. Hackeng TM, van 't Veer C, Meijers JC, Bouma BN. Human protein S inhibits prothrombinase complex activity on endothelial cells and platelets via direct interactions with factors Va and Xa. J Biol Chem. 1994;269:21051-21058
11. Heeb MJ, Rosing J, Bakker HM, Fernandez JA, Tans G, Griffin JH. Protein S binds to and inhibits factor Xa. Proc Natl Acad Sci U S A. 1994;91:2728-2732
12. van 't Veer C, Hackeng TM, Biesbroeck D, Sixma JJ, Bouma BN. Increased prothrombin activation in protein S-deficient plasma under flow conditions on endothelial cell matrix: an independent anticoagulant function of protein S in plasma. Blood. 1995;85:1815-1821
13. van Wijnen M, Stam JG, van't Veer C, Meijers JC, Reitsma PH, Bertina RM, Bouma BN. The interaction of protein S with the phospholipid surface is essential for the activated protein C-independent activity of protein S. Thromb Haemost. 1996;76:397-403

14. van 't Veer C, Butenas S, Golden N, Mann K. Regulation of prothrombinase activity by protein S. Thromb Haemost. 1999;82:80-87.
15. Dahlbäck B, Stenflo J. High molecular weight complex in human plasma between vitamin K-dependent protein S and complement component C4b-binding protein. Proc Natl Acad Sci U S A. 1981;78:2512-2516
16. Dahlbäck B. Inhibition of protein Ca cofactor function of human and bovine protein S by C4b-binding protein. J Biol Chem. 1986;261:12022-12027
17. Sere KM, Janssen MP, Willems GM, Tans G, Rosing J, Hackeng TM. Purified protein S contains multimeric forms with increased APC-independent anticoagulant activity. Biochemistry. 2001;40:8852-8860
18. Gandrille S, Borgel D, Ireland H, Lane DA, Simmonds R, Reitsma PH, Mannhalter C, Pabinger I, Saito H, Suzuki K, Formstone C, Cooper DN, Espinosa Y, Sala N, Bernardi F, Aiach M. Protein S deficiency: a database of mutations. For the Plasma Coagulation Inhibitors Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. Thromb Haemost. 1997;77:1201-1214
19. Leroy-Matheron C, Gouault-Heilmann M, Aiach M, Gandrille S. A mutation of the active protein S gene leading to an EGF1-lacking protein in a family with qualitative (type II) deficiency. Blood. 1998;91:4608-4615
20. Gandrille S, Aiach M. Identification of mutations in 90 of 121 consecutive symptomatic French patients with a type I protein C deficiency. The French INSERM Network on Molecular Abnormalities Responsible for Protein C and Protein S deficiencies. Blood. 1995;86:2598-2605
21. Rezende SM, Lane DA, Mille-Baker B, Samama MM, Conard J, Simmonds RE. Protein S Gla-domain mutations causing impaired Ca(2+)-induced phospholipid binding and severe functional protein S deficiency. Blood. 2002;100:2812-2819
22. van Wijnen M, van 't Veer C, Meijers JC, Bertina RM, Bouma BN. A plasma coagulation assay for an activated protein C-independent anticoagulant activity of protein S. Thromb Haemost. 1998;80:930-935
23. Nicolaes GA, Thomassen MC, van Oerle R, Hamulyak K, Hemker HC, Tans G, Rosing J. A prothrombinase-based assay for detection of resistance to activated protein C. Thromb Haemost. 1996;76:404-410
24. Rosing J, Tans G, Govers-Riemslag JW, Zwaal RF, Hemker HC. The role of phospholipids and factor Va in the prothrombinase complex. J Biol Chem. 1980;255:274-283
25. Persson KE, Hillarp A, Dahlbäck B. Analytical considerations for free protein S assays in protein S deficiency. Thromb Haemost. 2001;86:1144-1147
26. Gruber A, Griffin JH. Direct detection of activated protein C in blood from human subjects. Blood. 1992;79:2340-2348

27. Mitchell CA, Jane SM, Salem HH. Inhibition of the anticoagulant activity of protein S by prothrombin. J Clin Invest. 1988;82:2142-2147
28. Heeb MJ, Griffin JH. Activated protein C-dependent and -independent anticoagulant activities of protein s have different structural requirements. Blood Cells Mol Dis. 2002;29:190-199
29. Smirnov MD, Safa O, Esmon NL, Esmon CT. Inhibition of activated protein C anticoagulant activity by prothrombin. Blood. 1999;94:3839-3846
30. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood. 1996;88:3698-3703

Chapter 6

Effect of oral contraceptives on the anticoagulant activity of protein S in plasma

Koenen RR, Thomassen MC, Tans G, Rosing J, and Hackeng TM.

Summary

We determined anticoagulant parameters that depend on protein S function in plasma *i.e.* the APC-independent anticoagulant activity of protein S (expressed as pSR) and APC resistance determined with thrombin generation-based tests (expressed as nAPCsr) as well as plasma levels of total and free protein S and prothrombin in men, women not using oral contraceptives (OC), and in women using second or third generation OC. Thrombin generation in the APC resistance assays was initiated either with factor Xa (Xa-APCsr) or tissue factor (TF-APCsr). The APC-independent anticoagulant activity of protein S was highest in men (pSR=1.69) and gradually decreased from women not using OC (pSR=1.49) via women using second generation (pSR=1.35) to third generation OC (pSR=1.27). The pSR correlated inversely with nAPCsr determined with the tissue factor-based APC resistance test (TF-APCsr) but not with nAPCsr determined with the factor Xa-based assay (Xa-APCsr). Multiple linear regression analysis in which sex, OC use, and protein S and prothrombin levels were included as independent variables and the pSR, TF-APCsr or Xa-APCsr as dependent variables indicated that plasma protein S levels poorly predict the pSR and the TF-APCsr, but are the main determinant of the Xa-APCsr. This indicates that OC use alters the expression of protein S activity. This phenomenon can be caused by differences in modulation of the activity of protein S by other plasma proteins that change during OC use or by OC-induced changes in the protein S molecule that impair its anticoagulant activity.

Introduction

The mechanisms by which the vitamin K-dependent anticoagulant protein, protein S regulates blood coagulation are complex and not completely understood. The best characterized function of protein S is its ability to act as a cofactor of activated protein C (APC) in the inactivation of activated factors V (FVa)[1] and VIII (FVIIIa)[2,3]. In addition, protein S has been shown to exhibit anticoagulant activity in the absence of APC, an activity which may involve direct interactions with activated factor X (FXa), phospholipids, FVa and FVIIIa[4-7]. In several studies, the APC-independent anticoagulant activity of protein S has been shown to be expressed in plasma[8,9] and recently, a new assay for quantification of this activity in plasma was developed[10].

The importance of protein S as an anticoagulant is emphasized by the severe clinical manifestations associated with protein S-deficiency[11-13]. Although several studies indicate protein S-deficiency is a risk factor for venous thromboembolism (VTE)[14,15], the association between protein S and VTE remains difficult to establish for several reasons. Protein S levels vary with age[16], gender[17] and hormonal status[18]; men have higher protein S levels than women[15,18,19] and the use of certain kinds of oral contraceptives (OC) has been shown to cause a decrease in protein S levels[16,17,20]. In addition, *quantification of plasma protein* S levels is complicated by the fact that approximately 60% of protein S present in plasma circulates in complex with C4b binding protein (C4BP)[21], a component of the classical complement cascade. Measurements of free protein S appear to depend on the method used and incorrect sample-handling may lead to overestimation[22].

Protein S function is also subject to various influences. Complexation of protein S with C4BP renders protein S inactive as cofactor of APC[23] in FVa inactivation but both the free and the C4BP-complexed form of protein S have been reported to act as cofactor in APC-mediated FVIIIa inactivation[24]. The APC-independent activity of protein S is only mildly affected by complexation with C4BP[4,10] but high levels of prothrombin, such as those associated with the prothrombin G20210A mutation, appear inhibitory particularly in plasmas with reduced protein S levels[10].

Oral contraceptive (OC) use is associated with an increased risk of venous thrombosis, but the mechanism behind this risk increase is still not completely understood. It has been proposed that acquired APC resistance may explain the risk observed in women using OC. APC resistance as determined in a thrombin generation-based test is strongly modulated by plasma protein S levels presumably due to the resulting variation of APC cofactor activity. In this paper we have further explored the relationship between OC use and protein S function in plasma. The APC-independent anticoagulant activity and the antigen levels of protein S (both total and free protein S) were determined in plasma of men, women not using OC and in women using second or third generation OC. In addition, plasma levels of prothrombin, an important independent thrombotic risk factor[25,26] as well as a modulator of the APC-independent anticoagulant activity of protein S[10] were also determined. The APC cofactor activity of protein S was indirectly assessed with thrombin generation-based APC resistance assays in which prothrombin activation was initiated in plasma by the addition of factor Xa or tissue factor in the absence and presence of added APC.

Materials and methods

Bovine serum albumin (BSA, clinical reagent grade) and HEPES were purchased from ICN (Costa Mesa, CA, USA). Protein S and factor Xa were purchased from Enzyme Research Laboratories (South Bend, IN, USA). 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Rabbit polyclonal antibodies against protein S were obtained from DAKO (Glostrup, Denmark). Immunopure TMB substrate kit was purchased from Pierce (Rockford, IL, USA). Ecarin™ was obtained from Pentapharm (Basel, Switzerland). The purified fibrinogen converting enzyme Ancrod from the Malayan pit viper (*Agkistrodon rhodostoma*) was obtained from the National Institute for Biological Standards and Control (NIBSC, Hertfordshire, UK). Other reagents were of the highest grade available.

Preparation of plasma samples

Normal pooled plasma was prepared as described[10]. The plasma samples used in this investigation were obtained by venipuncture and processed as described[10]. Subjects positive for factor V Leiden were omitted from the study. This resulted in a plasma collection of men (n=16, mean age 25.5 yrs), women not using OC (n=32, mean age 26.6 yrs), women using second generation OC (n=32, mean age 24.9 yrs) and third generation OC (n=29, mean age 24.3 yrs). Second generation OC contain ethinylestradiol and levonorgestrel and third generation OC contain ethinylestradiol and desogestrel or gestodene. Women not using OC were neither pregnant nor menopausal. Subjects on OC were using OC for at least four months and were not using other medication. The study was approved by the institutional medical ethics committee, informed consent for additional scientific research was given by all participants. Protein S-depleted plasma was prepared as described[10] and contained about 10 % of residual protein S.

Purification of beta-chain containing C4BP

C4BP was isolated from 20 units of freshly frozen plasma (5 L) as previously described[27].

Determination of protein S antigen levels

Protein S antigen levels were determined using pooled normal plasma as standard and were expressed as IU/ml assuming that pooled normal plasma contained 1 IU/ml. Total protein S antigen was determined with an ELISA using rabbit-polyclonal antibodies (DAKO) against human protein S and was performed essentially as described[28], except that samples were incubated in the microtiter plate overnight at room temperature.

Free protein S concentrations were determined by ELSA as described by Giri et al.[29] and plasma samples were processed as described by Persson et al.[22]. Briefly, purified C4BP was coated on a microtiter plate and incubated for one hour with 1:1250 dilutions of sample plasma in 25 mM Hepes, pH=7.7, 175 mM NaCl, 5 mM $CaCl_2$ and 0.5% BSA. After washing with the same buffer to remove unbound protein, bound protein S was quantified using 1:1000 diluted horse radish peroxidase conjugated rabbit polyclonal antibodies against protein S and visualization using the TMB substrate kit (Pierce). Absorbance was measured at 450 nm in a Biorad plate reader.

Determination of plasma prothrombin

Plasma prothrombin concentrations were determined using the purified prothrombin activator Ecarin™ from the venom of the saw-scaled viper (*Echis carinatus*) as described[30].

Preparation of phospholipid vesicles

Phospholipid vesicles were prepared by sonication as described before[10]. The DOPS/DOPC (20/80, M/M) vesicle suspension was stored in aliquots under nitrogen at -80°C.

APC-independent anticoagulant activity of protein S

The one stage Xa-based assay used to quantify the APC-independent anticoagulant activity of protein S was performed on an ACL-300 (Instrumentation Laboratory, Milan, Italy) as previously described[10]. Briefly, plasma (100 µl) was incubated with 12 µl DAKO rabbit polyclonal anti-protein S antibodies (2.5 µM final concentration) or 12 µl of 25 mM Hepes, pH=7.7, 175 mM NaCl for 30 minutes at room temperature. Coagulation was initiated in 50 µl of the (non-)treated plasma with 150 µl of 0.025 nM factor Xa, 8 µM DOPS/DOPC (20/80, M/M) vesicles in 25 mM Hepes, pH=7.7, 175 mM NaCl containing 11 mM $CaCl_2$ and 5% BSA. This resulted in final concentrations of 0.02 nM factor Xa, 6 µM DOPS/DOPC (20/80, M/M) vesicles, 5 mM $CaCl_2$ and 5% (w/v) BSA. The clotting time was defined as the time needed for the absorbance to pass a threshold value of 0.06 OD units above the initial absorbance of the sample (onset of coagulation). The ability of protein S to prolong the clotting time was expressed as protein S ratio (pSR) i.e. the ratio of the clotting times determined in the absence and presence of anti-protein S antibodies.

APC resistance measurements

APC resistance was determined by measurement of the effect of added APC on thrombin generation initiated in plasma with either tissue factor[31-33] or factor Xa. Briefly, plasma was defibrinated with 1.5 U/ml Ancrod using a plastic spatula. After 10 minutes at 37 °C, the spatula containing adhered fibrin was discarded resulting in defibrinated plasma. To 80 µl defibrinated plasma incubated at 37°C was added 45 µl starting solution containing tissue factor or factor Xa, $CaCl_2$ and phospholipid vesicles, with or without APC to result in final concentrations of 0.64 ng/ml tissue factor or 0.44 nM factor Xa, 15 µM phospholipid (PS/PE/PC; 20/20/60; M/M/M) and 16 mM added $CaCl_2$ with or without 5 nM APC.

After 20 min incubation at 37°C the amount of α_2-macroglobulin-thrombin (α_2M-IIa) complexes was determined with S2238 by measuring the rate of change in absorbance determined at 405 nm at 37°C in a microtiter plate reader (SLT) set in the kinetic mode[33]. The reaction conditions were chosen such that residual thrombin formation in normal plasma in the presence of APC was approximately 10% of that determined in the absence of APC (cf. also ref. [33]). APC sensitivity ratios in subject plasmas were obtained by calculation of the ratio of the amounts of α_2M-IIa determined in the presence and absence of APC and normalized against the same ratio determined in the same experiment in normal plasma (formula 1, cf. [34]):

$$nAPCsr = \frac{(\alpha_2\text{M-IIa}_{+APC}/\alpha_2\text{M-IIa}_{-APC})_{\text{plasma sample}}}{(\alpha_2\text{M-IIa}_{+APC}/\alpha_2\text{M-IIa}_{-APC})_{\text{normal plasma}}} \quad (1)$$

Statistical methods

Differences between population means were tested by Mann-Whitney test for multiple group comparison using SPSS. To assess possible determinants of the pSR, TF-APCsr and Xa-APCsr, multiple linear regression analysis was performed with pSR, TF-APCsr and Xa-APCsr as dependent variable and levels of total protein S, free protein S and prothrombin as independent variables. The resulting non standardized regression coefficient B for a coagulation factor indicates the change in the pSR, TF-APCsr or Xa-APCsr, when that particular coagulation factor increases with one unit and all other variables in the model are unchanged. To investigate the influence of age, sex, and oral contraceptive use, multiple linear regression analysis was performed with sex (0=male, 1=female), age, second generation OC (0=non user, 1=user) and third generation OC (0=non user, 1=user) as additional independent variables.

Results

Effect of OC use on plasma protein S and prothrombin levels.

Figure 1 shows protein S antigen (Protein S_{total}, Fig. 1A and Protein S_{free}, Fig. 1B) and prothrombin levels (Fig. 1C) in men, women not using OC and in women using second or third generation OC. The mean values for each group are summarized in Table 1. Both total and free protein S levels in men were significantly higher than in women who were not using OC. Protein S_{total} and protein S_{free} antigen levels were the same for women who were not using OC and who were using second generation OC, but were significantly lower in third generation OC users.

Prothrombin levels were similar in men (1.00 ± 0.12 IU/ml) and non-OC users (0.98 ± 0.13). In plasmas of users of second and third generation OC the levels of prothrombin were elevated ($p<0.005$) to a similar extent (1.08 ± 0.10 IU/ml and 1.11 ± 0.12 IU/ml, respectively, Table 1).

Table 1: Protein S and prothrombin levels in men, non users, second and third generation OC using women (mean±SD).

population	Total protein S (IU/ml)	Free Protein S (IU/ml)	Prothrombin (IU/ml)
men (n=16)	0.95 ± 0.08	1.05 ± 0.13	1.00 ± 0.12
women no OC (n=32)	0.84 ± 0.12	0.87 ± 0.18	0.98 ± 0.13
2nd generation OC (n=32)	0.83 ± 0.13	0.93 ± 0.16	1.08 ± 0.10
3rd generation OC (n=29)	0.72 ± 0.10	0.74 ± 0.16	1.11 ± 0.12

Significant differences between populations are displayed in Figure 1.

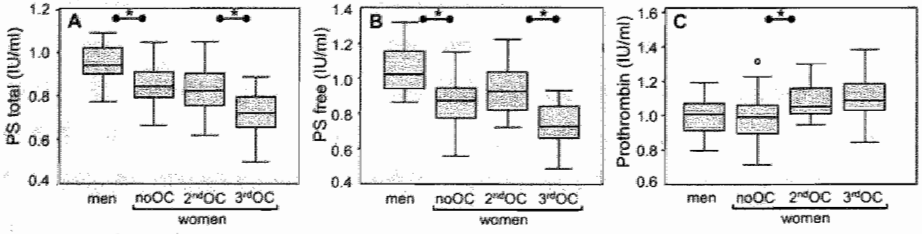

Figure 1. Protein S and prothrombin levels in plasmas from men, women not using OC and women using second or third generation OC.
Total protein S (A), free protein S (B) and prothrombin levels (C) in individuals were determined as described in Materials and Methods.
Statistical significance ($P<0.05$) between groups is indicated with an asterisk (*). Population medians are depicted as horizontal bars in the boxes. The boxes end at quartiles Q1 and Q3, respectively, the whiskers are extended to the farthest points that are not outliers (i.e., that are within 1.5 times the interquartile range of Q1 and Q3), the outliers are depicted as open dots.

Effect of OC use on parameters that probe the functional activity of protein S in plasma.

Since protein S has multiple anticoagulant activities, the effects of OC use on a number of haemostatic parameters that depend on the functional activity of protein S in plasma, *i.e.* its APC-independent anticoagulant activity and its APC cofactor activity, were determined.

The APC-independent anticoagulant activity of protein S was expressed as a clotting time ratio (pSR)[10], and the APC cofactor activity was expressed as APC sensitivity ratio (nAPCsr) using two thrombin generation-based APC-resistance tests. In these assays, thrombin generation was initiated by the addition of either factor Xa (Xa-APCsr) or tissue factor (TF-APCsr[31]). Figure 2 shows the strong influence of varying plasma protein S levels, achieved by mixing varying amounts of normal plasma with protein S-depleted plasma, on the pSR, Xa-APCsr, and TF-APCsr. The APC-independent anticoagulant activity of protein S (pSR), was the highest in men (pSR = 1.69 ± 0.12) and was significantly lower in women not using OC (pSR = 1.49 ± 0.08, $p<0.001$) (Fig. 3A), consistent with the lower antigen levels of protein S (Fig. 1AB, Table 1).

Although women using second generation OC had the same protein S antigen levels as women not using OC (Fig. 1AB) a further decrease in average pSR was observed in second generation OC users (pSR = 1.35 ± 0.08, p<0.001; Fig. 3A, Table 2).

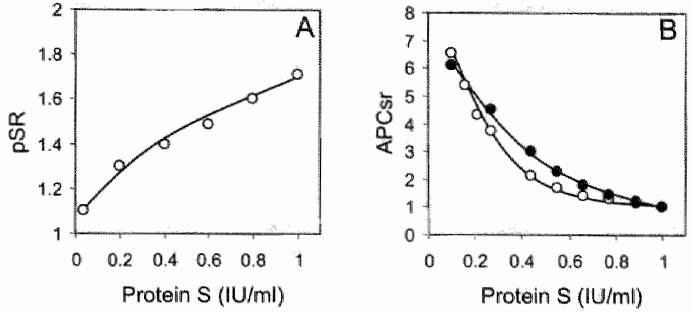

Figure 2. Effect of the plasma protein S level on the APC-independent protein S activity test and thrombin generation-based APC resistance tests. Protein S levels in plasma were varied by mixing protein S-depleted plasma with normal pooled plasma. **A**: Anticoagulant activity of protein S in the absence of APC (protein S Ratio: pSR) of the plasma mixtures were determined as a function of the protein S concentration as described. **B**: APC sensitivity ratios (APCsr) of the various plasma mixtures as a function of the protein S concentration were determined with thrombin-generation-based APC resistance tests in which thrombin formation was initiated with tissue factor (filled symbols) or factor Xa (open symbols) as described in the experimental procedures.

Third generation OC users showed an average pSR which, compared to women using second generation OC, was further decreased (pSR = 1.27 ± 0.07, p<0.001), a phenomenon that may at least partially be explained by the additional decrease in protein S levels observed in third generation OC users (p<0.001, Fig. 1 Table 1).

Table 2: pSR, TF-APCsr and Xa-APCsr in men, non users, second and third generation OC using women (mean±SD).

population	pSR	TF-APCsr	Xa-APCsr
men (n=16)	1.69 ± 0.12	0.97 ± 0.49	0.89 ± 0.25
women no OC (n=32)	1.49 ± 0.08	1.90 ± 0.53	1.42 ± 0.33
2nd generation OC (n=32)	1.35 ± 0.08	2.93 ± 0.56	1.40 ± 0.39
3rd generation OC (n=29)	1.27 ± 0.07	4.01 ± 0.87	1.70 ± 0.44

Significant differences between populations are displayed in Figure 2.

In the different populations, the TF-APCsr was inversely related to the APC-independent activity of protein S. In groups where the TF-APCsr was low, the pSR was high, and *vice versa* (Fig. 3AB). This inverse relation was less evident when APC resistance was determined with the factor Xa-based APC-resistance assay (Fig. 3AC). With this assay, there was no difference between the Xa-APCsr of second generation OC users and women not using OC.

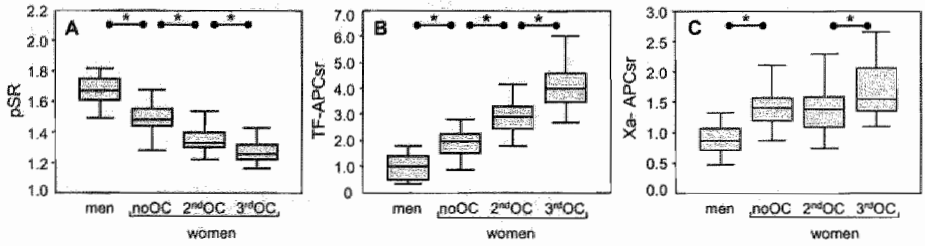

Figure 3. APC-independent anticoagulant activity of protein S and sensitivity to APC in plasma from men, women and second or third generation OC using women.
(A) APC-independent activity of protein S in individuals, represented as clotting time ratio (pSR) was determined as described in Materials and Methods. Differences between all groups were statistically significant (P<0.001).
(B) TF-APCsr were determined by quantifying the effect of APC on thrombin generation initiated in plasma with tissue factor as described in Materials and Methods. Differences between all groups were statistically significant (P<0.001).
(C) Xa-APCsr were determined by quantifying the effect of APC on thrombin generation initiated in plasma with factor Xa as described in Materials and Methods.
Statistical significance (P<0.05) between groups is indicated with an asterisk (*). Population medians are depicted as horizontal bars in the boxes, outliers are depicted as open dots. Vertical bars demarcate 1.5 times the interquartile range.

Multiple linear regression analysis

Multiple linear regression analysis of the three functional tests in which the pSR, TF-APCsr or Xa-APCsr were taken as dependent variable and total protein S, free protein S and prothrombin as independent variables (Model 1) showed that total as well as free protein S and prothrombin were determinants of the pSR and Xa-APCsr and that the total protein S and prothrombin levels were the main determinants of the TF-APCsr (Table 3). Adjustment for sex, age and oral contraceptive use (Model 2) reduced the importance of the protein levels as determinants, particularly in the assay that quantifies the APC-independent anticoagulant activity of protein S and in the tissue factor-based APC resistance test. In fact, total and free protein S became non-significant determinants for pSR and free protein S was non-significant for both the TF-APCsr and Xa-APCsr (Table 4). Sex appeared to be a determinant of all three functional tests and oral contraceptive use was a strong determinant of both the pSR and the TF-APCsr.

Table 3: Regression coefficients for APC resistance and the APC independent anticoagulant activity of protein S: MODEL 1

	pSR	TF-APCsr	Xa-APCsr
	B (95% C.I.)		
R^2	0.49	0.57	0.43
Protein S_{free} (U/L)	0.22(0.01 - 0.43)*	-1.30(-2.78 - 0.19)**	-0.77(-1.38 - -0.15)*
Protein S_{total} (U/L)	0.49(0.22 - 0.75)**	-4.83(-6.70 - -2.97)**	-1.20(-1.97 - -0.42)*
Prothrombin (U/L)	-0.59(-0.76- -0.42)**	4.34(3.12 - 5.57)**	1.17(0.66 - 1.68)**

*P<0.05, **P<0.001. Non standardized regression coefficients B for the variables, which were obtained after multivariate linear regression analysis, indicate the change in pSR, TF-APCsr or Xa-APCsr when that particular coagulation factor increases with one unit and all other variables in the model are unchanged. R^2 indicates the fraction by which the independent variables used in the regression model explain the test outcome.

Table 4: Regression coefficients for APC resistance and the APC independent anticoagulant activity of protein S: MODEL 2

	pSR	TF-APCsr	Xa-APCsr
	B (95% C.I.)		
R^2	0.77	0.78	0.49
Protein S_{free} (U/L)	0.13(-0.03 - 0.28)	-0.52(-1.68 - 0.65)	-0.45(-1.09 - 0.20)
Protein S_{total} (U/L)	0.11(-0.08 - 0.31)	-2.24(-3.69- -0.80)*	-1.28(-2.07 - -0.48)*
Prothrombin (U/L)	-0.20(-0.34 - -0.06)*	1.38(0.33 - 2.44)*	1.14(0.56 - 1.72)**
Age (year)	0.001(-0.002 -0.004)	0.004(-0.02 - 0.03)	0.02(0.003 - 0.03)*
Sex (m to f)	-0.16(-0.21 - -0.11)**	0.59(0.21 - 0.98)*	0.30(0.09 - 0.52)
2nd OC (use)	-0.13(-0.17 - -0.09)**	0.91(0.59 - 1.23)**	-0.10(-0.27 - 0.08)
3rd OC (use)	-0.17(-0.22 - -0.12)**	1.66(1.28 - 2.03)**	-0.02(-0.23 - 0.18)

*P<0.05, **P<0.001. Non standardized regression coefficients B, obtained after multivariate linear regression analysis, indicate the change in pSR, TF-APCsr or Xa-APCsr when an independent variable increases with one unit (total and free protein S and prothrombin) or with one year (age) or changes (male to female, OC use vs. non use) and all other variables in the model are unchanged. R^2 indicates the fraction by which the independent variables used in the regression model explain the test outcome.

Discussion

In this study we have further explored the effects of gender and oral contraceptive use on protein S levels in plasma and on the concomitant changes brought about in the efficacy by which the protein C system down-regulates thrombin formation. The overall effects of oral contraceptives on plasma protein S levels and on nAPCsr determined with the tissue factor-based thrombin generation test presented in this paper are in good agreement with earlier reported data[20,35]. Moreover, while this work was in progress, Kemmeren et al.[36] using an alternative assay[8], also showed that oral contraceptive use decreases the APC-independent anticoagulant activity of protein S.

The most interesting aspect of the findings reported here is that there is a discrepancy between protein S antigen levels and protein S function in plasma. This is particularly illustrated by the observation that, compared with non OC users, the APC-independent anticoagulant activity of protein S (pSR) is significantly decreased in second generation OC users while they have the same total and free protein S antigen levels as non-OC users.

Moreover, multiple linear regression analysis shows that protein S antigen levels in plasma become a minor determinant of the pSR when age, sex and OC use are included as independent variables (Table 4: Model 2) indicating that plasma protein S levels poorly predict its APC-independent anticoagulant activity. This discrepancy can be caused by the circulation of dysfunctional protein S molecules that are detected by the antigen assays but that are not active in a functional assay and/or by modulation of protein S activity by other plasma components that vary in the different individuals. With respect to the latter possibility, we recently reported that plasma prothrombin levels strongly modulate the APC-independent anticoagulant activity of protein S and that the pSR is decreased in plasmas of individuals carrying the prothrombin G20210A mutation[10]. Thus, the reduced APC-independent activity of protein S in second generation OC users, in the absence of a difference in protein S levels compared to non-users, can at least in part be explained by the increase in prothrombin levels observed in women using second generation OC. The further decrease of APC-independent anticoagulant activity of protein S observed in third generation OC users compared with second generation OC users, is likely due to the additional decrease in protein S levels.

Similar arguments apply to the thrombin generation-based APC resistance assays. The experiment presented in Figure 2 shows that plasma protein S is an indispensable cofactor for APC in these assays. Since the protein S/C4BP complex and thrombin-cleaved protein S are both inactive as cofactors of APC[23,37], the thrombin generation-based APC resistance assays will be affected by variations in the relative amounts of these protein S forms in plasma. But thrombin generation-based APC resistance assays are also sensitive to a wide variety of other factors such as the presence of the factor V Leiden mutation or the use of oral contraceptives. Furthermore, it is possible that modulatory mechanisms such as discussed above for the APC-independent anticoagulant activity may also contribute to the elevated APC-resistance found in OC users.

Thus, the APC-cofactor activity of protein S in OC users may be reduced by OC-induced changes in the plasma milieu such as elevated prothrombin levels. In that case the elevated prothrombin levels in OC users indirectly contribute to APC resistance by impairing the APC cofactor activity of protein S[38]. The between group variations in the APC-independent anticoagulant activity of protein S (pSR) show a strong inverse relationship with APCsr determined with the tissue factor-based APC resistance assay (Fig. 3A,B). Although these assays probe two distinct functional activities of protein S, this suggests that they share common determinants and that these determinants are influenced by OC use in the same manner. Moreover, the outcomes of these assays are less well predicted by protein S antigen than APC resistance determined with the Xa-based assay (Fig. 3C). The latter assay, in which thrombin generation is initiated directly with FXa (in the presence or absence of added APC), is expected to reflect almost solely the direct effect of the cofactor activity of protein S on the inactivation of factor Va by APC whereas the tissue factor-based assay may be influenced by additional plasma components, that may also have an effect on protein S activity.

The close inverse relationship for the differences between groups in the pSR and the (clinically validated) tissue factor-based APC resistance assay suggests that APC-independent effects of protein S may have been overlooked as a possible determinant contributing to the risk of venous-thrombosis associated with OC use. The APC-independent anticoagulant activity of protein S, in turn, is modulated by prothrombin levels and high prothrombin levels may considerably reduce the APC-independent activity of protein S, particularly in individuals with low protein S levels. The possibility of synergistic prothrombotic risks in the case of both high prothrombin levels and low protein S levels in individuals been reported before[39,40]. Moreover, high prothrombin levels (independent of the prothrombin G20210A mutation) have also been found to be an additional risk factor in users of OC[25]. We hypothesize that the reduction of the APC-independent and possibly also APC-cofactor activity of protein S at high prothrombin levels may provide a rationale for this increased risk.

References

1. Walker FJ. Regulation of activated protein C by a new protein. A possible function for bovine protein S. J Biol Chem. 1980;255:5521-5524
2. Walker FJ, Chavin SI, Fay PJ. Inactivation of factor VIII by activated protein C and protein S. Arch Biochem Biophys. 1987;252:322-328
3. Koedam JA, Meijers JC, Sixma JJ, et al. Inactivation of human factor VIII by activated protein C. Cofactor activity of protein S and protective effect of von Willebrand factor. J Clin Invest. 1988;82:1236-1243
4. Hackeng TM, van 't Veer C, Meijers JC, et al. Human protein S inhibits prothrombinase complex activity on endothelial cells and platelets via direct interactions with factors Va and Xa. J Biol Chem. 1994;269:21051-21058
5. Heeb MJ, Mesters RM, Tans G,et al. Binding of protein S to factor Va associated with inhibition of prothrombinase that is independent of activated protein C. J Biol Chem. 1993;268:2872-2877
6. Heeb MJ, Rosing J, Bakker HM, et al. Protein S binds to and inhibits factor Xa. Proc Natl Acad Sci U S A. 1994;91:2728-2732
7. Koppelman SJ, Hackeng TM, Sixma JJ, et al. Inhibition of the intrinsic factor X activating complex by protein S: evidence for a specific binding of protein S to factor VIII. Blood. 1995;86:1062-1071
8. van Wijnen M, van 't Veer C, Meijers JC, et al. A plasma coagulation assay for an activated protein C-independent anticoagulant activity of protein S. Thromb Haemost. 1998;80:930-935
9. van 't Veer C, Hackeng TM, Biesbroeck D, et al. Increased prothrombin activation in protein S-deficient plasma under flow conditions on endothelial cell matrix: an independent anticoagulant function of protein S in plasma. Blood. 1995;85:1815-1821
10. Koenen RR, Tans G, van Oerle R, et al. The APC-independent anticoagulant activity of protein S in plasma is decreased by elevated prothrombin levels due to the prothrombin G20210A mutation. Blood. 2003;102:1686-1692
11. Mahasandana C, Suvatte V, Marlar RA, et al. Neonatal purpura fulminans associated with homozygous protein S deficiency. Lancet. 1990;335:61-62
12. Mahasandana C, Suvatte V, Chuansumrit A, et al.. Homozygous protein S deficiency in an infant with purpura fulminans. J Pediatr. 1990;117:750-753
13. Schwarz H, Fischer M, Hopmeier P, et al. Plasma protein S deficiency in familial thrombotic disease. Blood. 1984;64:1297-1300.
14. Comp PC, Nixon RR, Cooper MR, et al. Familial protein S deficiency is associated with recurrent thrombosis. J Clin Invest. 1984;74:2082-2088

15. Faioni E, Valsecchi C, Palla A, et al. Free protein S deficiency is a risk factor for venous thrombosis. Thromb Haemost. 1997;78:1343-1346.
16. Dykes AC, Walker ID, McMahon AD, et al. A study of Protein S antigen levels in 3788 healthy volunteers: influence of age, sex and hormone use, and estimate for prevalence of deficiency state. Br J Haematol. 2001;113:636-641
17. Boerger LM, Morris PC, Thurnau GR, et al. Oral contraceptives and gender affect protein S status. Blood. 1987;69:692-694
18. Liberti G, Bertina R, Rosendaal F. Hormonal state rather than age influences cut-off values of protein S: reevaluation of the thrombotic risk associated with protein S deficiency. Thromb Haemost. 1999;82:1093-1096.
19. Henkens CM, Bom VJ, Van der Schaaf W, et al. Plasma levels of protein S, protein C, and factor X: effects of sex, hormonal state and age. Thromb Haemost. 1995;74:1271-1275
20. Tans G, Curvers J, Middeldorp S, et al. A randomized cross-over study on the effects of levonorgestrel- and desogestrel-containing oral contraceptives on the anticoagulant pathways. Thromb Haemost. 2000;84:15-21
21. Dahlbäck B, Stenflo J. High molecular weight complex in human plasma between vitamin K-dependent protein S and complement component C4b-binding protein. Proc Natl Acad Sci U S A. 1981;78:2512-2516
22. Persson KE, Hillarp A, Dahlbäck B. Analytical considerations for free protein S assays in protein S deficiency. Thromb Haemost. 2001;86:1144-1147
23. Dahlbäck B. Inhibition of protein Ca cofactor function of human and bovine protein S by C4b-binding protein. J Biol Chem. 1986;261:12022-12027
24. van de Poel RH, Meijers JC, Bouma BN. C4b-binding protein inhibits the factor V-dependent but not the factor V-independent cofactor activity of protein S in the activated protein C-mediated inactivation of factor VIIIa. Thromb Haemost. 2001;85:761-765
25. Legnani C, Cosmi B, Valdre L, et al. Venous thromboembolism, oral contraceptives and high prothrombin levels. J Thromb Haemost. 2003;1:112-117
26. Poort SR, Rosendaal FR, Reitsma PH, et al. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood. 1996;88:3698-3703
27. Koenen RR, Gomes L, Tans G, et al. The Ser460Pro mutation in recombinant protein S Heerlen does not affect its APC-cofactor and APC-independent anticoagulant activities. Thromb Haemost. 2004;91:1105-1114
28. Sere KM, Janssen MP, Willems GM, et al. Purified protein S contains multimeric forms with increased APC-independent anticoagulant activity. Biochemistry. 2001;40:8852-8860

29. Giri TK, Hillarp A, Hardig Y, et al. A new direct, fast and quantitative enzyme-linked ligandsorbent assay for measurement of free protein S antigen. Thromb Haemost. 1998;79:767-772
30. Bertina RM, van der Marel-van Nieuwkoop W, Loeliger EA. Spectrophotometric assays of prothrombin in plasma of patients using oral anticoagulants. Thromb Haemost. 1979;42:1296-1305
31. Rosing J, Tans G, Nicolaes GA, et al. Oral contraceptives and venous thrombosis: different sensitivities to activated protein C in women using second- and third-generation oral contraceptives. Br J Haematol. 1997;97:233-238
32. Curvers J, Thomassen MC, Nicolaes GA, et al. Acquired APC resistance and oral contraceptives: differences between two functional tests. Br J Haematol. 1999;105:88-94
33. Curvers J, Christella M, Thomassen LG, et al. Effects of (pre-)analytical variables on activated protein C resistance determined via a thrombin generation-based assay. Thromb Haemost. 2002;87:483-492
34. de Ronde H, Bertina RM. Laboratory diagnosis of APC-resistance: a critical evaluation of the test and the development of diagnostic criteria. Thromb Haemost. 1994;72:880-886
35. Mackie IJ, Piegsa K, Furs SA, et al Protein S levels are lower in women receiving desogestrel-containing combined oral contraceptives (COCs) than in women receiving levonorgestrel-containing COCs at steady state and on cross-over. Br J Haematol. 2001;113:898-904
36. Kemmeren JM, Algra A, Meijers JC, et al. Effect of second- and third-generation oral contraceptives on the protein C system in the absence or presence of the factor VLeiden mutation: a randomized trial. Blood. 2004;103:927-933
37. Borgel D, Gaussem P, Garbay C, et al. Implication of protein S thrombin-sensitive region with membrane binding via conformational changes in the gamma-carboxyglutamic acid-rich domain. Biochem J. 2001;360:499-506
38. Tran S, Norstrom E, Dahlbäck B. Inhibition by prothrombin of individual activated protein C-mediated cleavages in factor Va. Journal of Thrombosis and Haemostasis. 2003;1 Supplement 1 July:P1055
39. Boinot C, Borgel D, Kitzis A, et al. Familial thrombophilia is an oligogenetic disease: involvement of the prothrombin G20210A, PROC and PROS gene mutations. Blood Coagul Fibrinolysis. 2003;14:191-196
40. Castaman G, Tosetto A, Cappellari A, et al. The A20210 allele in the prothrombin gene enhances the risk of venous thrombosis in carriers of inherited protein S deficiency. Blood Coagul Fibrinolysis. 2000;11:321-326

Chapter 7

Summary and discussion

Summary and discussion

Deficiency of the vitamin K-dependent anticoagulant protein S is a risk factor for venous and possibly also arterial thrombosis. Protein S has a molar mass of 75 kD, is heavily posttranslationally modified and consists of various domains that are important for its function. Approximately 60% of protein S in plasma exists in complex with C4b-binding protein (C4BP), a component of the classical complement system. Protein S exerts its anticoagulant effect in plasma via two main mechanisms: on one side, it is a cofactor for activated protein C (APC) and on the other side protein S possesses anticoagulant activity of its own. The APC-cofactor function of protein S is best characterized. Protein S accelerates the APC-catalyzed degradation of activated coagulation factors V and VIII (factors Va and VIIIa). The complex of protein S with C4BP is not active as APC-cofactor in the inactivation of factor Va, but retains APC-independent anticoagulant activity, just like free protein S. In the absence of APC, protein S inhibits prothrombin activation via direct interactions with factors Xa and Va that are bound to phospholipid membranes provided by platelets and endothelial cells. The nature of the various interactions has not been exactly determined and most data regarding these interactions have been generated from experiments in reconstituted systems in which purified components (e.g. proteins, phospholipids) have been used. Not much is known about the physiological importance and mode of action of protein S in plasma in the absence of APC.

The research presented in this thesis focuses on the APC-independent activity of protein S. Central goals were the determination of factors important for the APC-independent anticoagulant effect of protein S. The aforementioned reconstituted systems enabled the investigation of potentially important modulatory factors residing in protein S itself, e.g. via mutagenesis studies. Measurements of the APC-independent activity of protein S in plasma allowed us to identify factors outside of protein S. The nature and determinants of the APC-independent activity should, at least essentially, be equal in the case of plasma and reconstituted systems.

Data obtained from measurements in plasma and reconstituted systems were integrated into a hypothetical model which describes the APC-independent regulation of prothrombin activation by protein S. In case there is a discrepancy between data obtained in reconstituted systems and plasma, a lead for future investigations may be provided.

Characterization of recombinant and plasma-purified protein S Heerlen.

Based on previously reported data[1], structural determinants specific for the APC-independent anticoagulant activity of protein S may be located in the SHBG-like domain of protein S. In this respect, the naturally occurring Heerlen mutation was interesting to investigate. The Heerlen variant of protein S is the consequence of a point mutation in the DNA, resulting in the substitution of a serine for a proline residue at position 460 in the SHBG-like region of protein S. Because of this mutation, asparagine 458 is not glycosylated, but the mutation may also have implications for the structure of the affected region.

The protein S Heerlen mutation has been associated with low levels of free protein S, but normal levels of total protein S, although the direct involvement of the Ser 460 Pro mutation itself in causing these low levels is still controversial. Despite the decreased plasma concentrations of free protein S that have been reported to accompany the protein S Heerlen mutation, individuals who carry this mutation do not have a significantly increased risk for thrombosis. It has been reported that protein S Heerlen has decreased APC-cofactor activity in the inactivation of factor Va Leiden[2] although the pathophysiological consequence of this observation is not clear since epidemiological data obtained from individuals who carry both the factor Va Leiden and the protein S Heerlen mutations is not yet available.

Because the APC-cofactor activities of normal protein S and protein S Heerlen in the APC-catalyzed inactivation of normal factor Va are the same, an increased APC-independent activity of protein S Heerlen may provide a mechanism for the compensation of the decreased plasma levels of protein S. This was the working hypothesis on which the work presented in chapters 3 and 4 was based. In order to test this hypothesis, the Heerlen mutation was inserted into the cDNA of protein S. Protein S Wild Type (WT) and protein S Heerlen were expressed in human embryonic kidney (HEK) 293 cells and isolated from the conditioned culture media. In chapter 3, the biochemical characterization of the purified recombinant protein S preparations is described. Protein S Heerlen and protein S WT were compared in terms of mass, binding to C4BP, APC-independent activity and APC-cofactor activities in the inactivation of factors Va, Va Leiden and VIIIa. Recombinant protein S Heerlen was identical in all its studied aspects to recombinant protein S WT; no abnormal (gain of) function of recombinant protein S Heerlen was found. For unknown reasons, these results are in contrast with the previous report in which an impaired APC-cofactor activity of protein S Heerlen specifically in the inactivation of factor Va Leiden is described[2].

Although the findings described above show that no differences exist between the activities of recombinant protein S Heerlen and WT, carriers of the protein S Heerlen mutation were found to have an increased APC-independent activity of protein S (chapter 4). This prompted us to isolate protein S from the plasmas of protein S Heerlen carriers and to compare this with plasma-purified normal protein S. It was found that the complex of C4BP and protein S Heerlen (protein S Heerlen/C4BP), purified from plasma of heterozygous carriers, possessed a 7 to 22-fold increased factor Xa inhibitory activity in the presence or absence of factor Va respectively, compared with normal protein S/C4BP. This increased activity of protein S Heerlen/C4BP was explained by the observation that protein S Heerlen/C4BP displayed increased binding to purified factor Xa. Furthermore, protein S Heerlen/C4BP, added to plasma, prolonged the clotting time 14 times more effectively than normal protein S/C4BP.

However, the difference in activity between free protein S purified from plasma of normal individuals or carriers of the protein S Heerlen mutation was considerably less and moreover, little difference was found between the APC-independent anticoagulant activities of recombinant protein S WT and protein S Heerlen, either reconstituted with C4BP or not.

Thus, the protein S Heerlen/C4BP complex isolated from plasma from individuals carrying the protein S Heerlen mutation appears to be the species responsible for the observed elevated APC-independent anticoagulant activity in prothrombinase assays and in plasma. On the basis of these observations we propose that the elevated APC-independent anticoagulant activity of the protein S Heerlen-C4BP complex may compensate for the decreased plasma levels of protein S in carriers of the protein S Heerlen mutation and may explain why these individuals are not exposed to an increased risk of venous thrombosis.

With respect to the difference in anticoagulant activities of normal protein S/C4BP and protein S Heerlen/C4BP it should be emphasized that protein S Heerlen/C4BP complex purified from plasma of heterozygous carriers still contains approximately 50% normal protein S. Therefore the real difference between the APC independent anticoagulant activities protein S/C4BP and protein S Heerlen/C4BP will be even larger than observed in this study.

The observations that protein S in complex with C4BP exhibits a higher anticoagulant activity than free protein S and that the protein S-C4BP complex purified from plasma of carriers of the Heerlen mutation is more anticoagulant than the complex purified from normal plasma deserve further discussion.

Several explanations can be provided for the observation that protein S and protein S Heerlen in complex with C4BP displayed a higher APC-independent activity than free protein S WT and protein S Heerlen. It is possible that free protein S loses APC-independent activity during the purification from plasma or conditioned medium: it has been reported that protein S is able to undergo changes during purification.

For example, protein S has been shown to self-aggregate in the presence of EDTA, a reagent commonly used in protein purifications[3,4]. C4BP may play a protective role in such purification procedures in a sense that the APC-independent function of protein S is retained. It is also possible that a conformational change in protein S by binding to C4BP or an enhanced affinity of the protein S-C4BP for the prothrombinase complex renders the protein S-C4BP complex a better inhibitor of prothrombin activation.

Even more interesting were the observations that 1) protein S present in plasma from heterozygous carriers of the protein S Heerlen mutation exhibited a higher APC-independent anticoagulant activity (higher pSR) than protein S present in plasma of normal individuals and 2) that, in reconstituted systems containing purified proteins, the protein S Heerlen/C4BP complex purified from plasma of heterozygous carriers of the protein S Heerlen mutation was a better APC-independent anticoagulant than normal protein S/C4BP. In this respect it should be mentioned that affinity purification using a monoclonal antibody rendered protein S significantly more active as an APC-independent anticoagulant, a phenomenon that was explained by an *in vitro* induced conformational change[5]. Such an altered conformation might already be present in the protein S Heerlen/C4BP complex and may explain the higher affinity of protein S Heerlen/C4BP for immobilized factor Xa, compared with protein S WT/C4BP. Another explanation for the increased APC-independent activity of protein S Heerlen/C4BP complex in plasma and in model systems may be that protein S and/or the protein S/C4BP complex require an additional plasma component that potentiates their APC-independent anticoagulant activity. An increased affinity of protein S Heerlen/C4BP for such an as hypothetical plasma component and co-purification of this component during the isolation of the protein S Heerlen/C4BP complex can explain both the increased pSR of protein S Heerlen plasma and the enhanced APC-independent anticoagulant activity of the purified protein S Heerlen-C4BP complex in model systems.

Taken together, the data presented in chapters 3 and 4 emphasize that additional studies are required to fully characterize protein S Heerlen. It needs to be confirmed in a larger population that plasmas from of protein S Heerlen carriers indeed have an elevated APC-independent activity, compared with plasmas of controls. In such studies, carriers of protein S Heerlen who are also positive for factor V Leiden may also be included to investigate whether protein S Heerlen and factor V Leiden indeed show synergistic prothrombotic effects, as postulated previously[2].

Plasma with low levels of protein S correspondingly have low APC-independent anticoagulant activity and hence a significantly lower average pSR of 1.31 ± 0.09 was found in a group of 16 protein S-deficient individuals. During the development of the assay it was found that the plasma prothrombin concentration is inversely related to the APC-independent anticoagulant activity of protein S. In a group of 46 individuals that carry the prothrombin G20210A mutation, a mutation that is associated with elevated levels of prothrombin, the APC-independent anticoagulant activity of protein S (1.47 ± 0.21) was significantly lower than that of the control group. This observation may, at least in part, provide an explanation for the elevated risk for venous thrombosis associated with the prothrombin G20210A mutation. The influence of factors other than protein S on the described assay must not necessarily be regarded as a disadvantage. Although specificity of an assay for the analyte is a prerequisite for any functional assay, it may come at the cost of "research potential" in a sense that determinants residing in the plasma environment may remain unnoticed. This concept is supported by the identification of prothrombin as a modulator of the APC-independent anticoagulant activity of protein S using the described test that is inherently sensitive for determinants other than just antigen levels.

In chapter 6, protein S levels and protein S function (i.e. APC-cofactor and APC-independent activity) were investigated in men, women not using oral contraceptives and women using second or third generation oral contraceptives. The APC-independent anticoagulant activity of protein S was determined using the plasma assay described in chapter 5 and the APC-cofactor function was estimated using two thrombin generation based APC resistance assays initiated with tissue factor and factor Xa respectively. APC resistance was expressed as normalized ratios: TF-APCsr (tissue factor-triggered) or Xa-APCsr (factor Xa-triggered) and both ratios strongly depend on protein S levels when determined in an experiment in which protein S-depleted plasma was mixed with normal plasma. APC-independent activity of protein S, expressed as pSR, was significantly higher in men (pSR=1.69) than in women not using oral contraceptives (pSR=1.49).

In women using second and third generation oral contraceptives, the pSR significantly further decreased to 1.35 and 1.27, respectively. Interestingly, the APC resistance in the four groups under investigation, determined in a thrombin generation-based assay in which tissue factor was used as a trigger, appeared to be inversely related with the APC-independent activity of protein S. In groups were APC resistance was high, the APC-independent activity of protein S was low, and *vice versa*. This inverse relation was less pronounced when APC resistance was measured using factor Xa as coagulation initiator in the APC resistance test. The presence of an apparent inverse relation between TF-APCsr and pSR indicates that the APC-cofactor and APC-independent activities of protein S have common determinants.

Multiple linear regression analysis was performed in which age, sex, oral contraceptive use, and protein S and prothrombin levels were included as independent variables and pSR, TF-APCsr, and Xa-APCsr as dependent variables. This multiple regression analysis indicated that the APC-independent activity of protein S is not predicted by protein S total and free levels but rather by sex, oral contraceptive use and, to a lesser extent, by prothrombin levels. The TF-APCsr was poorly predicted by protein S levels contrary to the Xa-APCsr, which was strongly determined by protein S total levels and also by plasma prothrombin levels. This difference may originate from the more direct effect that factor Xa has on the initiation of thrombin formation. Upon addition, factor Xa may be incorporated into the prothrombinase complex instantaneously. Therefore, the anticoagulant effect of protein S measured in the factor Xa-triggered APC resistance assay may directly reflect its APC-cofactor function in the inactivation of factor Va.

From the observations discussed in this section it can be concluded that the activity of protein S is, at least in part, influenced by environmental factors in plasma. The difference in APC-independent activity of protein S between men and women not using oral contraceptives can be explained by decreased protein S levels observed in women.

The difference between women not using oral contraceptives and women using second generation oral contraceptives can only in part be explained by the increased prothrombin levels found in women using second generation oral contraceptives. A new type of functional deficiency may be postulated from these data: a plasma protein with a normal concentration and integrity may be functionally impaired through influences from its plasma environment. Having introduced this concept, the long lasting controversy around protein S as a risk factor for thrombosis may be approached from this alternative perspective: in clinical/epidemiological studies, protein S function as APC cofactor or -independent anticoagulant should be determined in combination with antigen levels rather than antigen levels alone.

The quantitation of protein S levels in plasma is complicated by the existence of the protein S/C4BP complex. Low free protein S levels have been reported to be a more sensitive measure of venous thrombotic risk[8] and free protein S levels are often measured using assays that rely on binding of free protein S to C4BP or to monoclonal antibodies specific for free protein S. But although the affinity of the protein S/C4BP interaction is very high, the equilibrium of free and complexed protein S may still depend on additional factors (e.g. temperature) and rearrangement of this equilibrium may have a strong influence on free protein S measurements. Indeed, the amount of free protein S measured has been shown to depend on sample handling, assay temperature and assay principle[9,10]. In addition, protein S levels in plasma vary significantly with age, gender and hormonal status[11,12]. It is therefore difficult to define unambiguous cut-off values for protein S-deficiency. For example, a link between protein S-deficiency and venous thrombosis could not be found when cut-off values were adjusted for hormonal status[13].

It may be clear that an undisputable link between protein S-deficiency and thrombosis can only be established when a consensus about the precise definition of protein S-deficiency is achieved. With the identification of modulating plasma factors outside of protein S, another dimension may be added to this problem. In order to clearly establish protein S as a risk factor for venous thrombosis, also additional knowledge may be necessary about factors in plasma that influence protein S activity. Antigen levels alone may be insufficient to clearly correlate protein S with the occurence of venous and arterial thrombosis.

APC-independent anticoagulant activity of protein S in plasma: proposed mode of action.

In literature, significant APC-independent anticoagulant effects of protein S in plasma have consistently been found[6,14,15]. In purified systems on the other hand, the discovery of multimeric protein S and the weak inhibition of prothrombin activation by multimer-free protein S[4] has led to questions about the clinical or physiological significance of the direct inhibition of prothrombin activation by protein S. The observed APC-independent inhibition in plasma [6,14,15], indicates that the mode of action of protein S in the plasma environment likely consists of more facets than only inhibition of prothrombin activation via direct interactions with components that comprise the prothrombinase complex.

These proposed multiple modes of action of protein S in plasma are summarized in figure 1. Protein S may exert its function in plasma either by acting as a direct intrinsic tenase inhibitor[16,17] or protein S may inhibit prothrombin activation directly or indirectly via the interaction with an yet to be identified component. This component may directly inhibit prothrombin activation and protein S may enhance this inhibition (Fig. 1). Conversely, the weak direct prothrombinase inhibition by protein S observed in purified systems [4,18] may be enhanced in plasma by the unidentified component (Fig.1).

The inhibition of prothrombin activation may be localized on apoptotic cells, inflamed endothelium and platelet aggregates (Fig. 1) and protein S may thereby maintain the hemostatic equilibrium and may thus serve to locally prevent thrombotic events by preventing the very onset of thrombin generation. The inhibitory effect of prothrombin on this localized APC-independent inhibition of prothrombin activation by protein S may play an additional feedback regulatory role since local consumption of prothrombin may lead to a more pronounced effect of protein S. Furthermore, heparin has been shown to enhance the APC-independent activity of protein S in plasma[6] and heparin-like molecules (glycosaminoglycans) on the endothelial cell surface may thus potentiate the APC-independent activity of protein S or the protein S/C4BP complex (Fig. 1). In combination with the recent anti-inflammatory effects described for protein S and the protein S/C4BP complex[19-21], protein S may also play a role in the prevention of arterial thrombosis. Although some reports associate protein S-deficiency with arterial thrombosis[22-25], more extensive studies have to be performed to clearly establish a link between protein S-deficiency and arterial thrombosis. The plasma assay presented in this thesis may provide a useful tool for this purpose.

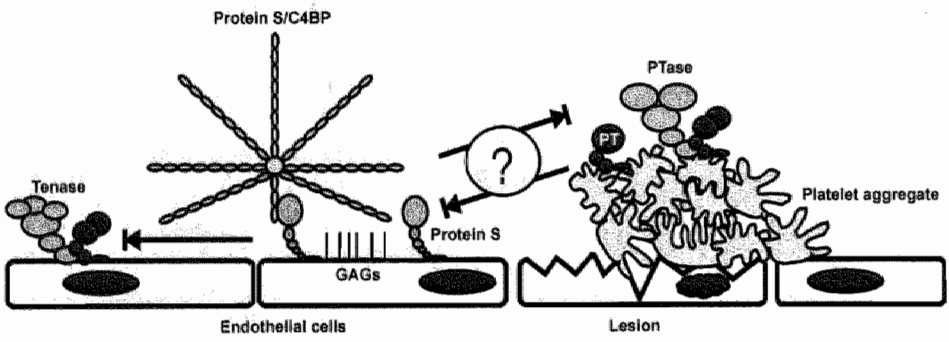

Figure 1: Schematic representation of APC-independent regulation of thrombin generation.
Protein S or the protein S/C4BP complex may inhibit the intrinsic tenase and prothrombinase complexes on inflamed endothelium or platelet aggregate. The inhibition of blood coagulation by protein S may be augmented by a yet to be identified plasma component and is negatively regulated by prothrombin, possibly via the same component. Glycosaminoglycans (GAGs) present on endothelial cells may also play a modulatory role in the inhibition of thrombin generation by protein S.

References

1. Heeb MJ, Kojima Y, Rosing J, Tans G, Griffin JH. C-terminal residues 621-635 of protein S are essential for binding to factor Va. J Biol Chem. 1999;274:36187-36192
2. Giri TK, Yamazaki T, Sala N, Dahlbäck B, de Frutos PG. Deficient APC-cofactor activity of protein S Heerlen in degradation of factor Va Leiden: a possible mechanism of synergism between thrombophilic risk factors. Blood. 2000;96:523-531
3. Pauls JE, Hockin MF, Long GL, Mann KG. Self-association of human protein S. Biochemistry. 2000;39:5468-5473
4. Sere KM, Janssen MP, Willems GM, Tans G, Rosing J, Hackeng TM. Purified protein S contains multimeric forms with increased APC-independent anticoagulant activity. Biochemistry. 2001;40:8852-8860
5. Mitchell C, Kelemen S, Salem H. The anticoagulant properties of a modified form of protein S. Thromb Haemost. 1988;60:298-304.
6. van Wijnen M, van 't Veer C, Meijers JC, Bertina RM, Bouma BN. A plasma coagulation assay for an activated protein C-independent anticoagulant activity of protein S. Thromb Haemost. 1998;80:930-935
7. Kemmeren JM, Algra A, Meijers JC, Tans G, Bouma BN, Curvers J, Rosing J, Grobbee DE. Effect of second- and third-generation oral contraceptives on the protein C system in the absence or presence of the factor VLeiden mutation: a randomized trial. Blood. 2004;103:927-933
8. Faioni E, Valsecchi C, Palla A, Taioli E, Razzari C, Mannucci P. Free protein S deficiency is a risk factor for venous thrombosis. Thromb Haemost. 1997;78:1343-1346.
9. Goodwin AJ, Rosendaal FR, Kottke-Marchant K, Bovill EG. A review of the technical, diagnostic, and epidemiologic considerations for protein S assays. Arch Pathol Lab Med. 2002;126:1349-1366
10. Persson KE, Hillarp A, Dahlbäck B. Analytical considerations for free protein S assays in protein S deficiency. Thromb Haemost. 2001;86:1144-1147
11. Boerger LM, Morris PC, Thurnau GR, Esmon CT, Comp PC. Oral contraceptives and gender affect protein S status. Blood. 1987;69:692-694
12. Henkens CM, Bom VJ, Van der Schaaf W, Pelsma PM, Sibinga CT, de Kam PJ, van der Meer J. Plasma levels of protein S, protein C, and factor X: effects of sex, hormonal state and age. Thromb Haemost. 1995;74:1271-1275
13. Liberti G, Bertina R, Rosendaal F. Hormonal state rather than age influences cut-off values of protein S: reevaluation of the thrombotic risk associated with protein S deficiency. Thromb Haemost. 1999;82:1093-1096.

14. Koenen RR, Tans G, van Oerle R, Hamulyak K, Rosing J, Hackeng TM. The APC-independent anticoagulant activity of protein S in plasma is decreased by elevated prothrombin levels due to the prothrombin G20210A mutation. Blood. 2003;102:1686-1692
15. van 't Veer C, Hackeng TM, Biesbroeck D, Sixma JJ, Bouma BN. Increased prothrombin activation in protein S-deficient plasma under flow conditions on endothelial cell matrix: an independent anticoagulant function of protein S in plasma. Blood. 1995;85:1815-1821
16. Koppelman SJ, van't Veer C, Sixma JJ, Bouma BN. Synergistic inhibition of the intrinsic factor X activation by protein S and C4b-binding protein. Blood. 1995;86:2653-2660
17. Koppelman SJ, Hackeng TM, Sixma JJ, Bouma BN. Inhibition of the intrinsic factor X activating complex by protein S: evidence for a specific binding of protein S to factor VIII. Blood. 1995;86:1062-1071
18. Koenen RR, Gomes L, Tans G, Rosing J, Hackeng TM. The Ser460Pro mutation in recombinant protein S Heerlen does not affect its APC-cofactor and APC-independent anticoagulant activities. Thromb Haemost. 2004;91:1105-1114
19. Webb JH, Blom AM, Dahlback B. The binding of protein S and the protein S-C4BP complex to neutrophils is apoptosis dependent. Blood Coagul Fibrinolysis. 2003;14:355-359
20. Webb JH, Blom AM, Dahlback B. Vitamin K-dependent protein S localizing complement regulator C4b-binding protein to the surface of apoptotic cells. J Immunol. 2002;169:2580-2586
21. Anderson HA, Maylock CA, Williams JA, Paweletz CP, Shu H, Shacter E. Serum-derived protein S binds to phosphatidylserine and stimulates the phagocytosis of apoptotic cells. Nat Immunol. 2003;4:87-91
22. Allaart CF, Aronson DC, Ruys T, Rosendaal FR, van Bockel JH, Bertina RM, Briet E. Hereditary protein S deficiency in young adults with arterial occlusive disease. Thromb Haemost. 1990;64:206-210
23. Girolami A, Simioni P, Lazzaro AR, Cordiano I. Severe arterial cerebral thrombosis in a patient with protein S deficiency (moderately reduced total and markedly reduced free protein S): a family study. Thromb Haemost. 1989;61:144-147
24. Erli LC, Grandi R, Brambilla A, Zadra M, Felice B, Miele F, Finazzi G. Cerebral ischemia in a patient with protein S deficiency and carotid stenosis. Ital J Neurol Sci. 1994;15:121-123
25. Thommen D, Buhrfeind E, Felix R, Sulzer I, Furlan M, Lammle B. [Hemostasis parameters in 55 patients with venous and/or arterial thromboembolisms]. Schweiz Med Wochenschr. 1989;119:493-499

Samenvatting

Samenvatting

Proteïne S is een vitamine K-afhankelijk anticoagulant bloedplasma-eiwit. Proteïne S-deficiëntie is een risicofactor voor veneuze en mogelijk ook voor arteriële trombose. Proteïne S heeft een massa van 75 kD, is sterk posttranslationeel gemodificeerd en bestaat uit diverse domeinen die belangrijk zijn voor zijn functie. Ongeveer 60% procent van het proteïne S in plasma circuleert in complex met C4b-bindings eiwit (C4BP), een component van het klassieke complementsysteem. De anticoagulante werking van proteïne S is tweeledig: enerzijds is het een cofactor voor geactiveerd proteïne C (APC), anderzijds bezit proteïne S zelfstandige anticoagulante activiteit. De APC-cofactor functie van proteïne S is het best gekarakteriseerd. Proteïne S versnelt de door APC gekatalyseerde afbraak van de geactiveerde bloedstollingsfactoren V en VIII (factoren Va en VIIIa). Het complex van proteïne S met C4BP is niet actief als APC-cofactor in de inactivering van factor Va, maar heeft net als vrij proteïne S wel APC-onafhankelijke anticoagulante activiteit. In afwezigheid van APC remt proteïne S het protrombinase complex via directe interacties met factoren Xa en Va op fosfolipidenmembranen zoals die van bloedplaatjes of endotheelcellen. De aard van de diverse interacties is nog niet precies bepaald en de meeste data betreffende deze interacties zijn verkregen door middel van experimenten in gereconstitueerde systemen waarin gebruik wordt gemaakt van gezuiverde componenten. Over het effect en het belang van de APC-onafhankelijke anticoagulante activiteit van proteïne S in plasma is nog weinig bekend.

Het onderzoek beschreven in dit proefschrift concentreert zich op APC-onafhankelijke activiteit van proteïne S. Doelstellingen die centraal staan zijn het vaststellen van de determinanten die bepalend zijn voor de APC-onafhankelijke anticoagulante functie van proteïne S. In bovengenoemde gezuiverde systemen werden potentieel belangrijke factoren die eigen zijn aan proteïne S onder andere via mutagenese studies onderzocht.

Metingen van de APC-onafhankelijke activiteit van proteïne S in plasma stelden ons in staat ook invloedsfactoren te ontdekken die buiten proteïne S zelf liggen. De aard en de determinanten van de APC-onafhankelijke activiteit van proteïne S zouden, in ieder geval in essentie, gelijk moeten zijn voor plasma en voor gereconstitueerde systemen. Data verkregen uit metingen in plasma en resultaten uit gereconstitueerde systemen werden geïntegreerd tot een hypothetisch model waarin de APC-onafhankelijke regulatie van protrombine activering door proteïne S beschreven wordt. In het geval dat de data verkregen uit gereconstitueerde systemen niet overeenkomen met de data uit metingen in plasma, kan dit een aanknopingspunt leveren voor de verdere ontrafeling van het mechanisme van de APC-onafhankelijke remming van de bloedstolling door proteïne S.

In hoofdstuk 3 en 4 wordt een natuurlijke variant van proteïne S gekarakteriseerd, namelijk proteïne S Heerlen. De Heerlen-variant van proteïne S is het gevolg van een puntmutatie in het DNA, waardoor het aminozuur serine op positie 460 vervangen wordt door een proline. Hierdoor ontbreekt in ieder geval een glycosylerings sequentie bij asparagine 458 wat echter niet uitsluit dat de mutatie ook gevolgen kan hebben voor de structuur in het aangetaste gebied. Individuen die de proteïne S Heerlen mutatie dragen hebben verlaagde plasma concentraties van (met name vrij) proteïne S, maar desondanks geen noemenswaardig verhoogd risico op trombose. Er is gerapporteerd dat proteïne S Heerlen een verlaagde APC-cofactor activiteit heeft m.b.t. de APC-gekatalyseerde inactivatie van factor Va Leiden, maar de pathofysiologische consequentie van deze waarneming is nog niet duidelijk omdat epidemiologische data van individuen die zowel de factor V Leiden mutatie en de proteïne S Heerlen mutatie dragen nog niet beschikbaar zijn. Aangezien de APC-cofactor activiteit van proteïne S Heerlen niet verschilt van normaal proteïne S in individuen met normaal factor V, is het in dit geval mogelijk dat een verhoogde APC-onafhankelijke activiteit van proteïne S Heerlen het negatieve effect van de lage plasmawaarden compenseert. Om deze hypothese te testen werd de Heerlen mutatie aangebracht in het cDNA van proteïne S.

Zowel normaal proteïne S (WT) en proteïne S Heerlen werden tot expressie gebracht in menselijke embryonale HEK293 niercellen en de eiwitten werden uit de geconditioneerde kweekmedia geïsoleerd. De biochemische karakterisatie van de gezuiverde proteïne S preparaten is beschreven in hoofdstuk 3. Proteïne S Heerlen en proteïne S WT werden vergelijken m.b.t. massa, binding aan C4BP, APC-onafhankelijke activiteit en APC-cofactor activiteit in de door APC gekatalyseerde inactiveringen van factoren Va en VIIIa. Recombinant proteïne S Heerlen was identiek aan recombinant proteïne S WT; geen afwijkende (versterkte) functie van recombinant proteïne S Heerlen werd gevonden. Dit in tegenstelling tot de eerdere publicatie waarin een verlaagde APC-cofactor activiteit gevonden werd voor proteïne S specifiek in de inactivering van factor Va Leiden. Naar aanleiding van deze waarnemingen kon gesteld worden dat verlaagde plasmawaarden van proteïne S alleen, zoals bij de proteïne S Heerlen mutatie, niet leiden tot een verhoogd risico op veneuze trombose. Een andere mogelijkheid is dat een niet geïdentificeerde versterkende plasma-component een sterkere wisselwerking heeft met proteïne S Heerlen.

Ondanks de afwezigheid van verschillen tussen recombinant proteïne S Heerlen en proteïne S WT, zoals hierboven beschreven, werd in hoofdstuk 4 van dit proefschrift beschreven dat proteïne S in dragers van de proteïne S Heerlen mutatie een verhoogde APC-onafhankelijke activiteit uitoefende. Naar aanleiding van deze waarnemingen werd proteïne S geïsoleerd uit plasma van de proteïne S Heerlen dragers en vergeleken met proteïne S gezuiverd uit normaal plasma. Er werd gevonden dat het complex van C4BP en proteïne S Heerlen (proteïne S Heerlen/C4BP), gezuiverd uit plasma van heterozygote dragers, een 7-22 keer versterkte factor Xa remmende activiteit had dan het normale proteïne S/C4BP. Deze verhoogde activiteit van proteïne S Heerlen/C4BP kon verklaard worden met de waarneming dat proteïne S Heerlen/C4BP een versterkte binding vertoonde aan gezuiverd factor Xa. Tevens verlengde proteïne S Heerlen/C4BP, gezuiverd uit plasma, de stoltijd 14 keer effectiever dan normaal proteïne S/C4BP, indien toegevoegd aan plasma.

Echter, het verschil tussen plasma gezuiverd vrij proteïne S Heerlen en normaal proteïne S was aanzienlijk minder en ook recombinant proteïne S WT en proteïne S Heerlen, al dan niet in aanwezigheid van C4BP, vertoonden weinig verschil in antistollende activiteit.

Een mogelijke verklaring voor deze schijnbaar tegenstrijdige bevindingen zou kunnen zijn dat proteïne S tijdens de zuiveringsprocedure uit plasma of geconditioneerd medium van cellen zijn APC-onafhankelijke functie verliest. C4BP zou in deze zuiveringsprocedures wellicht een beschermende rol kunnen spelen zodat de APC-onafhankelijke functie van proteïne S behouden blijft. Ook de aanwezigheid van een niet eerder geïdentificeerde component zou de in hoofdstuk 4 beschreven waarnemingen kunnen verklaren. Geconcludeerd kan worden dat de verhoogde APC-onafhankelijke activiteit van proteïne S in plasma's van proteïne S Heerlen dragers en de verhoogde factor Xa en protrombinase remmende activiteit van proteïne S Heerlen/C4BP gezuiverd uit plasma als compensatie kunnen dienen voor de verlaagde proteïne S waarden in plasma's van proteïne S Heerlen dragers.

In hoofdstuk 5 werd een stoltest beschreven voor de kwantitering van de APC-onafhankelijke activiteit in plasma. Deze is gebaseerd op een eerder gepubliceerde stoltest voor de APC-onafhankelijke werking van proteïne S. De stoltijd van een plasmamonster in de aanwezigheid van een neutraliserend antilichaam tegen proteïne S werd vergeleken met de stoltijd van het onbehandelde plasmamonster. De ratio van deze stoltijden (proteïne S Ratio, pSR) is een directe maat voor de APC-onafhankelijke activiteit van proteïne S in plasma en neemt toe met toenemende APC-onafhankelijke activiteit. Het bleek dat de pSR vrijwel onafhankelijk was van de gebruikte concentratie fosfolipiden en dat ook het proteïne S/C4BP complex APC-onafhankelijke activiteit in plasma bezat.

De gemiddelde pSR voor een populatie van 60 gezonde individuen bedroeg 1.62 ± 0.16 en dit kwam goed overeen met de pSR van het referentie plasma (1.63 ± 0.08) dat een mengsel is van de plasma's van de gezonde individuen. Plasma met een laag gehalte aan proteïne S heeft dientengevolge ook een lage APC-onafhankelijke anticoagulante activiteit en zo werd dan ook een significant lagere gemiddelde pSR van 1.31 ± 0.09 gevonden in een groep van 16 proteïne S-deficiënte individuen. Tijdens het ontwikkelen van de test is gevonden dat de plasma protrombine concentratie de APC-onafhankelijke werking van proteïne S negatief beïnvloedt. In een groep van 46 individuen met de protrombine G20210A mutatie, die tot verhoogde protrombine concentraties in plasma leidt, is met behulp van de test gevonden dat deze een significant verlaagde APC-onafhankelijke activiteit van proteïne S (1.47 ± 0.21) hebben ten opzichte van de controlegroep. Dit zou een, in ieder geval gedeeltelijke, verklaring kunnen geven voor het verhoogde risico op trombose geassocieerd met de protrombine G20210A mutatie.

Indien onderzocht met de proteïne S plasma test bleken gebruiksters van de tweede en derde generatie anticonceptiepil een verlaagde APC-onafhankelijke activiteit van proteïne S te hebben, die gedeeltelijk verklaard werd met een verhoging van de plasma protrombine concentraties (hoofdstuk 6). Bij gebruiksters van de tweede generatie orale contraceptiva werd een significante daling van de APC-onafhankelijke antistollende activiteit waargenomen t.o.v. niet gebruiksters bij identieke plasmaconcentraties van vrij en totaal proteïne S. De activiteit van proteïne S was vergelijkbaar met een populatie van proteïne S-deficiënte patiënten. Een extra verlaging van proteïne S activiteit in gebruiksters van de derde generatie orale contraceptiva kon worden verklaard door een daling in proteïne S spiegels t.o.v. gebruiksters van de tweede generatie anticonceptiepil. De APC resistentie werd eveneens bepaald in de plasma's van gebruiksters van orale contraceptiva en tevens in mannen en vrouwen die de anticonceptiepil niet gebruikten.

Het bleek dat er tussen de groepen een omgekeerd verband bestond tussen APC resistentie (met als sterke determinant de APC cofactor functie van proteïne S) en de APC-onafhankelijke activiteit van proteïne S; in groepen waar de gemiddelde APC resistentie hoog was, was de gemiddelde APC-onafhankelijke activiteit laag, en andersom. Dit duidt erop dat deze anticoagulante functies van proteïne S gemeenschappelijke determinanten hebben. Een interessante bevinding was bovendien dat de concentraties van proteïne S in plasma van weinig voorspellende waarde waren voor zijn APC-onafhankelijke en cofactor functies in plasma.

Uit deze bevindingen en de bevindingen gerapporteerd in hoofdstuk 5 kan geconcludeerd worden dat de activiteit van proteïne S voor een deel beïnvloed wordt door omgevingsfactoren in het plasma, en zo aanleiding kan geven tot een nieuw type deficiëntie: een plasma eiwit met een normale concentratie en integriteit kan functioneel negatief worden beïnvloed door de plasma omgeving. In dit opzicht is het raadzaam om tijdens klinische of epidemiologische studies niet alleen de concentraties, maar ook de functionele activiteit van proteïne S in plasma te bepalen. De in dit proefschrift gepresenteerde proteïne S-plasmatest is hiervoor een nuttig gereedschap.

List of publications

Koenen RR, Tans G, van Oerle R, Hamulyak K, Rosing J, Hackeng TM. The APC-independent anticoagulant activity of protein S in plasma is decreased by elevated prothrombin levels due to the prothrombin G20210A mutation. *Blood.* 2003;102:1686-1692

Koenen RR, Gomes L, Tans G, Rosing J, Hackeng TM. The Ser460Pro mutation in recombinant protein S Heerlen does not affect its APC-cofactor and APC-independent anticoagulant activities. *Thromb Haemost.* 2004;91:1105-1114

Heeb MJ, Koenen RR, Fernandez JA, Hackeng TM. Direct anticoagulant activity of protein S-C4b binding protein complex in Heerlen heterozygotes and normals. *J. Thromb. Haemost.* 2004; 2:1766-73.

Koenen RR, Thomassen MC, Tans G, Rosing J, and Hackeng TM. Effect of oral contraceptives on the anticoagulant activity of protein S in plasma. *Accepted for publication.*

Accepted abstracts

XVIII Congress of The International Society on Thrombosis and Haemostasis, Paris 2001

Heeb MJ, Fernandez JA, Koenen RR, Hackeng TM. Direct anticoagulant activity of normal and Heerlen variant complexed protein S. *Thromb. Haemost.* 2001; Supplement July: OC101

XIX Congress of The International Society on Thrombosis and Haemostasis, Birmingham 2003

Koenen RR, Tans G, van Oerle R, Hamulyak K, Rosing J, Hackeng TM. Functional impairment of protein S in carriers of the prothrombin G20210A mutation. *J. Thromb. Haemost.* 2003; 1 Supplement 1 July: OC056.

Dankwoord

Dit proefschrift is niet volledig zonder diegenen te bedanken die me tijdens de afgelopen jaren hebben bijgestaan op deze lange weg naar wetenschappelijke bekwaamheid. Dit is geen gemakkelijke opgave; het schrijven van je dankwoord vereist mijns inziens net zoveel creativiteit als het doen van onderzoek. Ik ben dan ook bang dat ik degenen tekort ga doen in dit dankwoord omdat mijn dankbaarheidsgevoel groter is dan ik in twee pagina's kan verwoorden.

Als eerste wil ik Tilman bedanken. Er was nog nooit een begeleider (en die zal er denk ik ook niet meer komen) met wie ik zo'n geweldige omgang had als met jou. Ik vind dat alle kwaliteiten van een goede wetenschapper in jou aanwezig zijn: behalve scherp en volledig inzicht (in alles waarmee je je bezig houd), gedrevenheid en goed en fair leiderschap ook nog een enorme creativiteit. Ik kon altijd met alles bij jou aankomen, van flatlines bij de prothrombinase assays tot piepende remblokjes bij de auto. Je was altijd geïnteresseerd en bereidwillig om tijd voor me vrij te maken.

Jan, ik ben vreselijk trots dat ik op jouw lab onder jouw leiding ben opgeleid. Er werd altijd de hoogst realiseerbare wetenschappelijke kwaliteit nagestreefd en ik heb altijd heel hard mijn best gedaan om mijn werk tot die hoge standaard te tillen (ook al zou je dit aan de hand van mijn labjournaals niet direct zeggen). Je hebt een heerlijk nuchtere no-nonsense kijk op de zaken en ik hoop dat ik deze kijk een beetje heb overgenomen. Behalve de wetenschappelijke kwaliteit heb je ook een heel gezellige sfeer op jouw lab weten te scheppen en ik mis het bediscussieren van de data onder het genot van en pilsje, ook al dronk ik niet altijd mee. Vooral de laatste maanden van mijn aanstelling heb ik heel veel aan je hulp gehad, bedankt.

Guido, ondanks je vele verantwoordelijkheden buiten het onderzoek ben je onmisbaar binnen de groep. Jouw veelzijdigheid is al vele keren te pas gekomen; makroprogrammeur, computertechnicus, statisticus, moleculair bioloog, (bio-)chemicus, vertrouwenspersoon en ook nog eens de poortwachter voor wetenschappelijke manuscripten. Voor mij was het altijd een hele geruststelling als jij de data in orde vond, want dan konden ze gewoon niet verkeerd zijn. Jouw nuchtere kijk op de dingen heeft mij altijd goed gedaan, net als die heerlijke verhalen die je op het lab vertelde als je met de honden naar Frankrijk was geweest.

Kristin, ik zeg altijd veel woorden waarbij ook af toe iets zinvols tussen zit, jij zegt weinig woorden die daarentegen altijd zinvol zijn. Wij hebben als "werkbankgenoten" relatief weinig met elkaar gesproken maar ik heb onze gesprekken altijd als aangenaam ervaren. Je hebt de afgelopen jaren onverstoord de hindernissen getrotseerd die in jouw weg zijn gekomen en hiervoor heb ik veel bewondering.

Gerry, jouw (terug)komst op het lab was voor mij een ware verrijking. Ik denk dat we wat interesses en opvattingen betreft behoorlijk op een lijn zitten en dat is natuurlijk altijd een groot voordeel als je veel bij elkaar in de buurt bent. Ik hoop dat onze paden elkaar weer spoedig kruisen.

Stella, jouw veelvoudige nut in de groep is niet in een paar regels uit te drukken en ik waag niet eens een poging tot... Het lab is simpelweg niet hetzelfde zonder jou en dat merkte je alleen al aan de woensdagen waarop je weg was.

Elke, vanaf de eerste dag een feest, niet alleen op het werk maar ook tijdens de diverse uitgaans avondjes heb ik in jou een goede vriendin gevonden. Ik mis jouw schaterlach, maar daarentegen zijn alle deuren op mijn nieuwe werkplek nog zonder dwinger. Ik ben je veel dank verschuldigd en ik hoop dat onze vriendschap nog lang stand houdt.

Betta, mijn bescheiden, lieve doch zeer getalenteerde ex-kamergenote. De gesprekken die ik met jou heb gehad en het vertrouwen dat jij altijd in me hebt gehad hebben me door verschillende crisismomenten geleid. Dankjewel.

Jeroen, ik heb altijd veel plezier gehad aan jouw aanwezigheid op het lab. Bovendien ben je een heel fijne kamergenoot geweest tijdens onze cursus- en congresreisjes. Veel succes in de laatste fase van je promotie.

José, ik heb meermaals van jouw grote ervaring profijt gehad. Meer nog heb ik veel plezier gehad aan onze gesprekken over duizend en één onderwerpen. Ondanks dat je slechts één lab verder verhuisd bent, heb ik dit toch als verlies ervaren.

Kristoffer, I really enjoyed the time you and I spent in and outside the lab. You fitted perfectly in our group and I regret that you had to leave again. I hope we get to work together again some day.

Joyce, het zonnetje van het lab, bedankt voor de leuke tijd en veel succes in het werk en het ouderschap. Vivian, ik vond de korte tijd die we samengewerkt hebben erg aangenaam, bedankt hiervoor. Nieuwkomers Lisbeth en Kenneth, heel veel succes in de komende jaren.

Mary Jo, thank you for our pleasant cooperation, its result has made this thesis complete. I am very pleased with the result and I hope we get to work together again some day. José Fernández, thank you for our nice cooperation.

Lucio, jouw superstrakke sensorgrammetjes stralen van het papier af in hoofdstuk 3, bedankt voor onze plezierige samenwerking. Bonno, bedankt voor jouw nuttige inbreng, ik hoop dat we ooit nog samen in één auteurslijst mogen staan.

Berry, jouw waarde wordt pas echt duidelijk als je in een groep werkt waar geen Berry is. Leon, altijd aangenaam in de omgang en bovendien een uitstekende wetenschapper. Henri, bedankt voor je hulp vooral in het begin van mijn promotieperiode. Niko, je stond altijd garant voor veel lol, jammer dat ik de laatste maanden van mijn promotie zo weinig tijd had om even met je te praten. Heidi, Petra, Lisette, Hanneke, Waander van Heerde, Chris Reutelingsperger, Theo Lindhout, Simone, Jeff Wolfs, Dave Dekkers, Marjan, Edouard Bevers en Paul Comfurius, ik was altijd welkom voor vragen van allerhande aard, bedankt voor jullie hulp. Wencke, ook jou reken ik tot de grote aanwinsten van het lab, ik hoop dat onze fijne samenwerking nog lang stand houdt.

Karly Hamulyák, René en Dave, zonder jullie had dit proefschrift er heel anders uitgezien, heel erg bedankt voor jullie hulp. Trees, ik kon altijd op jouw hulp rekenen en nog steeds is jouw deur open voor een leuk gesprek.

Synaps BV, Prof. Hemker en Suzette, bedankt dat ik tijd op jullie lab heb mogen doorbrengen, deze tijd is zeer waardevol gebleken. George en Rita, jammer genoeg is onze aangename samenwerking vooralsnog niet in een publicatie uitgemond, desondanks waardeer ik jullie inbreng.

Christian Weber, vielen Dank für dein Vertrauen und die stimulierende Arbeitsumgebung. Das Kardiovaskuläre Molekularbiologie-Team, vielen Dank für die freundliche Atmosphäre und die angenehme Zusammenarbeit.

Verder wil ik nog mijn vroegere begeleiders bedanken wiens inzet en vertrouwen zeer belangrijk voor mijn vorming zijn geweest: Jan de Vente, Marjan Markerink, Harry Steinbusch, Guido Swart, Leon van Kempen, Peter Bloemers en niet in de laatste plaats Jan van Luxemborg.

Pap&mam, ik denk dat dit proefschrift nooit had bestaan zonder jullie nooit aflatende steun en vertrouwen, en bovendien jullie vasthoudendheid om mij een fatsoenlijke opleiding te laten volgen. Jullie hebben me door de beslissende vroege schooljaren heengeleid en hierdoor ben ik beland waar ik nu ben, hiervoor ben ik jullie zeer dankbaar.

Jennie, als er bij wetenschappelijk onderzoek al slachtoffers vallen, dan zijn het zeer zeker de partners van de onderzoekers. Ik wil je daarom niet alleen bedanken voor het prachtige omslagontwerp, maar ook voor jouw eindeloze geduld voor je te vaak afwezige vriend. Dankjewel.

Curriculum vitae

De auteur van dit proefschrift werd op 30 juni 1976 als Robert Ryan Koenen geboren in het St. Gregoriusziekenhuis te Brunssum. Na het verlaten van de St. Jozef basisschool in Merkelbeek begon hij in het najaar van 1987 aan het Hoger Algemeen Voortgezet Onderwijs (HAVO) op het St. Janscollege te Hoensbroek. In 1993 werd, na het behalen van het diploma, aan de chemische laboratoriumopleiding begonnen op de toenmalige Hogeschool Heerlen. Als specialisatie werd gekozen voor Biochemie en hiervoor heeft hij een éénjarige afstudeerstage gevolgd op de Universiteit Maastricht bij de vakgroep Neuropsychiatrie, Neuropsychologie und Psychobiologie van Prof. Dr. H.W. Steinbusch en Prof. Dr. J. Jolles onder de direkte begeleiding van M. Markerink-van Ittersum en Dr. J. de Vente. De chemische laboratoriumopleiding werd in juni 1997 afgesloten met een diploma die hem toegang verleende tot de verkorte studie chemie op de Katholieke Universiteit Nijmegen, die hij dan ook begin september 1997 startte. Wederom werd de discipline biochemie gekozen en een éénjarige hoofdvakstage gevolgd op de vakgroep Biochemie van Prof. Dr. H.P. Bloemers onder de direkte supervisie van Dr. G.W. Swart en Dr. L.C. van Kempen. Op 31 augustus 1999 mocht hij zijn universitaire diploma in ontvangst nemen en op 27 september 1999 begon hij als assistent in opleiding (AIO) bij de vakgroep Biochemie van Prof. Dr. J. Rosing. Hier heeft hij vier jaar lang gewerkt aan het project "Characterization of the APC-independent anticoagulant activity of protein S" van Dr. T.M. Hackeng. Sinds 1 december 2003 is hij werkzaam als wetenschappelijk medewerker aan de RWTH Aachen in de vakgroep Kardiovaskuläre Molekularbiologie van Prof. Dr. C. Weber.